ERRORS IN THE DETERMINATION OF SERUM CHOLESTEROL

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SUMMARY.

Within the limits of the present study, the accuracy of determinations of cholesterol in human sera depends more on the purification of the cholesterol than on the colorimetric reaction used. The application of a variety of colorimetric reactions to an extract of non-saponifiable material from serum gives values as accurate as the more complicated digitonide procedures. The accuracy of the principal methods for serum cholesterol determination are discussed in detail. In methods for preparation of the non-saponifiable extract based on that of Abell, Levy, Brodie and Kendall (1952) simplicity of operation may be combined with accuracy.

It is suggested the following points be established during standardization of methods omitting saponification. Since determination of cholesteryl stearate seems to be complicated by its insolubility in some colorimetric reagents for cholesterol, and in view of the colour developed with some of these reagents and polyunsaturated acids, identical molecular extinction coefficients should be obtained for cholesterol, cholesteryl stearate and the mixed cholesteryl esters of serum before the method can be judged accurate. The absence of effect of other serum constituents should then be verified by comparison of values obtained by the method and by application of the colorimetric reagent to the non-saponifiable matter of serum. Internal standards should be prepared with methods using saponification. Cholesteryl acetate should not be used in the standardization of methods for serum cholesterol determination as its behaviour in many methods is not typical of the cholesteryl esters of serum.

INTRODUCTION.

Though the rôle of cholesterol in coronary disease and athero-sclerosis is subject to much controversy, the level of serum cholesterol is widely held to be the best single indication of the latent condition obtainable by chemical means (Lewis, Olmstedt, Page, Lawry, Mann, Stare, Hanig, Lauffer and Moore, 1957; Doyle, De Lalla, Baker, Heslin and Brown, 1957; Stare, 1958; Lowry and Barach, 1958; Keys and Anderson, 1960). It is conceivable that prediction of the clinical disease may eventually depend on formulae associating parameters for age, sex, blood pressure, blood coagulation factors, serum cholesterol and uric acid levels, composition of the fatty acids in certain tissue lipids, body weight and other factors. However, in spite of the extensive investigation of serum cholesterol levels there is considerable confusion over the accuracy of the various methods used and much of the potential value of studies of cholesterol in coronary disease

has been lost accordingly. This subject is discussed elsewhere (Brown, 1961a).

The great variety of methods proposed for serum cholesterol determination is due to the complications associated with determination of cholesteryl digitonide, the uncertainty concerning the effects of saponification on cholesterol determination (Bloor, 1916) and the lack of stability and sensitivity of the Liebermann-Burchard reaction (Zak and Ressler, 1955). Clinical biochemists in particular have proposed simple methods for determination of serum cholesterol and a number of colorimetric reactions have been developed and applied to primary solvent extracts of serum and even directly to serum (notably by Zlatkis, Zak and Boyle, 1953). These procedures have been widely adopted, although Zak and Ressler (1955) note that the absence of interfering substances has not always been verified. In this paper the degree of purification of serum cholesterol required for its accurate determination by a variety of oxidative colorimetric reactions is examined.

The separation of cholesterol from proteins is usually carried out with a solvent mixture which includes an alcohol (Drekter, Bernhard and Leopold, 1935; Cohen and Chargaff, 1940; Lovern, 1942). Colorimetric determination of the cholesterol in this unpurified extract may then be carried out (Bloor, 1916; Zak, Dickenman, White, Burnett and Cherney, 1954; Lovern, 1955) or the lipids may be saponified and the cholesterol in a petrol-ether or chloroform extract (non-saponifiable extract) determined (Bloor, 1915; Abell, Levy, Brodie and Kendall, 1952; Brown, 1959). If not determined at this stage the cholesterol may be further purified with digitonin (Csonka, 1916; Schoenheimer and Sperry, 1934; Sperry and Webb, 1950; Brown, 1961b). It is probable that precipitation of the cholesterol with bromine would yield a purer product (Anderson, 1927; Schwenk, Stevens and Altschul, 1959) but no method which uses this principle has been developed for its quantitative determination.

In this paper the digitonide procedure developed by the writer (Brown, 1961b) provides standard values against which is assessed the accuracy of a number of colorimetric reactions applied variously to serum, to its alcohol-ether extract and to its non-saponifiable extract. The reactions were chosen for their diverse natures, as well as for their usefulness, and they were modified to obtain the greatest amount of information on accurate serum cholesterol determinations. This investigation is complementary to those of Morris (1960) and Wright, Tonks and Allen (1960).

The extent to which cholesteryl esters complicate the accurate determination of total serum cholesterol is not generally understood and in this paper cholesteryl stearate is examined for errors it may introduce into the determination of serum cholesterol by methods which do not involve saponification.

Materials and Methods.

Purification of reagents.

Absolute ethanol (CSR) was rendered aldehyde-free by treatment with m-phenylenediamine hydrochloride (Trinder, 1952). Ethyl ether (Woolwich Elliott, anaesthetic grade)

was freshly distilled prior to use. Glacial acetic acid AnalaR was purified by treatment with CrO_3 according to MacIntyre and Ralston (1954). Cholesterol (BDH) was recrystallized four times from ethanol, filtered in hexane and dried *in vacuo*. The cholesteryl stearate (BDH) was found by chromatographic analysis to contain 99 p.c. ester.

Colorimetric reactions and modifications.

In the method of Zlatkis *et al.* (1953) the colorimetric reagent (0·1 p.c. ferric chloride hexahydrate and 1 p.c. acetic acid in sulphuric acid) was added to serum dispersed in acetic acid. Several workers (Langan, Durrum and Jencks, 1955; Rosenthal, Pfluke and Buscaglia, 1957; Zak *et al.*, 1954; Crawford, 1958) have reported that turbidity may develop in this reagent but in this laboratory the turbidity was only troublesome on hot days. It formed more slowly when Ajax sulphuric acid AR was used instead of AnalaR sulphuric acid and its appearance seemed to depend on the water content of the acid. The turbidity was assumed to be due to formation of the insoluble anhydrous ferric chloride from the hexahydrate. Increasing the acetic acid content of the reagent from 1 p.c. to 4 p.c. delayed the formation of the turbidity by about two hours without altering the course of the reaction. It should be noted that the change in concentration of the reagents present during colour development was trivial, being about 1 p.c. in the ratio of acetic to sulphuric acids. This modified reagent was prepared from purified acetic acid and AnalaR sulphuric acid. The reagent was applied directly to serum according to the instructions of Zlatkis *et al.* (1953) and to the dry alcohol-ether and non-saponifiable extracts according to the procedure for colour development described by Langan *et al.* (1955). Bubbles that frequently formed in the cuvettes were removed by centrifugation.

The colorimetric reagent of Rose, Schattner and Exton (1941) (modified Tschugaeff reagent) was applied to the dry residues from the alcohol-ether extract and the non-saponifiable extract. Contrary to Lovern (1955), who also applied the reagent to the residue from the alcohol-ether extract, optimal colour stability occurred after development at 50° C. for 10 minutes. Under these conditions less than 1 p.c. decrease in absorbance occurred within the 30 minutes following colour development when cholesterol standards were being used but the decrease was greater when the colorimetric reagent was applied to the residue from the alcohol-ether extract of serum. This effect was minimized by determining the absorbance promptly after colour development. A persistent difficulty in obtaining reproducible conditions for colour stability was variation in the quality of zinc chloride used in the colorimetric reagent. Rose *et al.* (1941) applied the reagent to cholesteryl digitonide and they found difficulty in bringing the digitonide into solution in the reagent which may account for the higher temperatures and longer heating period required for optimal colour stability in their laboratory.

The colorimetric reactions of Abell et al. (1952) *and Brown* (1959) were applied to the residues from the alcohol-ether and non-saponifiable extracts. The preparation of the non-saponifiable extract was based on the method of Abell *et al.* (1952). However, saponification was carried out at 65° C. for one hour instead of 37° C. for 55 minutes as originally specified in order to be certain that all the cholesteryl esters present were saponified. Clarity on this point was essential in view of the application of a digitonide procedure to the non-saponifiable extract. Relevant discussion of saponification of cholesteryl esters is given in detail elsewhere (Brown, 1961b).

The cholesteryl digitonide was determined with the Liebermann-Burchard reagent as modified by Abell *et al.* (1952). In several cases additional determinations were made with the reagent mixture of Zlatkis *et al.* (1953) premixed and brought to 60° C. for five minutes in the presence of the digitonide. There was no systematic variation in the values obtained with the two reagents and all digitonide information was pooled.

Colour development with cholesteryl stearate.

The colorimetric reaction of Zlatkis et al. (1953). Cholesteryl stearate was dissolved in 3 ml. of hot glacial acetic acid and 0·05 ml. of saline was added instead of serum before the addition of the colorimetric reagent.

The colorimetric reaction of Pearson, Stern and McGavack (1953) was used in the manner described by Leppänen (1956). The cholesteryl stearate was dissolved in 0·2 ml. of hot acetic acid and 0·2 ml. saline was added before colour development.

The other colorimetric reactions used were applied to dry films of cholesterol or cholesteryl stearate and the colour developed in the manner originally described.

Treatment of serum before colour development.

Each of 10 sera was treated in the following manner.

Determination of serum cholesterol in the presence of whole serum by the method of Zlatkis *et al.* (1953). Serum (free of bromide) was delivered into 3 ml. of purified glacial acetic acid with a 0·05 ml. constriction pipette (Elliotts, England). Portions of the 3 ml. of acid were rinsed in and out of the pipette after the initial delivery: 0·05 ml. of saline was added to the cholesterol standard dissolved in 3 ml. of glacial acetic acid.

Determination of cholesterol in the dry residue from the alcohol-ether extract. The colorimetric reactions of Langan *et al.* (1955), Abell *et al.* (1952), Rose *et al.* (1941) and Brown (1959), with the modifications described above, were applied to the dry extract. The extraction procedure was based on that of Boyd (1936): 15 ml. of ethanol were swirled in a 50 ml. volumetric flask during the dropwise addition of 2 ml. of serum; 25 ml. of ether were then added and the mixture was shaken vigorously to disperse the precipitate. The mixture was refluxed for about 10 minutes and after cooling it was made up to volume with ether. The proteins were centrifuged off and 1 ml. samples were taken for each colorimetric procedure except that of Abell *et al.* (1952) for which 2 ml. were taken. The same pipettes were used to deliver standard solutions of cholesterol (10 mg. p.c.). Solvents were removed under nitrogen.

Cholesterol determination in the non-saponifiable extract. The colorimetric procedures applied to the alcohol-ether extract were also applied to the non-saponifiable extract. A 5 ml. sample of the alcohol-ether extract was evaporated to dryness and 5 ml. of alcoholic KOH (Abell *et al.*, 1952) were added. After saponification at 65° C. for one hour, 10 ml. of hexane were added to the cold hydrolysate followed by 5 ml. of water. Wet ground glass stoppers were inserted in the tubes and the phases were separated in a centrifuge after about 30 seconds of mixing. Samples (5 ml.) of the hexane extract were evaporated to dryness and colour development was carried out on the residue with the procedure of Abell *et al.* (1952). The other colorimetric procedures were applied to the dry residue from 1 ml. aliquots of the hexane extract. Cholesterol standards were prepared from 500 mg. of cholesterol treated exactly as the cholesterol in the dry residues of the alcohol-ether extracts. A standard was run in parallel with each unknown through the entire procedure.

Cholesteryl digitonide. Residues from the hexane extract were redissolved in 1 : 1 ethanol-acetone mixture. The cholesterol was precipitated from 2 ml. aliquots of these solutions with 1·4 ml. of 0·5 p.c. digitonin in 50 p.c. ethanol by the method described elsewhere (Brown, 1961b). Standard preparations of cholesteryl digitonide were prepared from samples of cholesterol run through the entire procedure including the stage of saponification.

Statistical analysis.

To effect randomisation the same pipettes were used for each series of standard and unknown determinations. Determinations were made on duplicate or triplicate samples of each serum. An internal standard was run with each determination so that an independent

estimate was obtained for the serum cholesterol level. The data are tabulated in Table 1, in which each value shown is the mean of the independent, replicate estimates on the serum sample. Analysis of variance of matched groups was used (Edwards, 1946). The standard deviations of individual values in Table 1 are based on duplicate values only.

RESULTS.

Serum cholesterol.

In Table 1 are summarised the results of estimating serum cholesterol (a) in serum directly, (b) after isolation in the dry alcohol-ether extract, (c) after isolation in the dry alcohol-ether extract followed by isolation from a dry non-saponifiable extract, (d) after purification through (b) and (c) followed by precipitation with digitonin.

The values obtained by application of the various colorimetric reactions to the dry non-saponifiable extract did not vary significantly. As a group, these values did not differ significantly from those obtained with the digitonide procedure. The mean value obtained by each colorimetric reaction applied to the dry alcohol-ether extract was significantly higher (at the 5 p.c. level) than that obtained by application of the same colorimetric reaction to the dry non-saponifiable extract. The values obtained by the colorimetric reactions of Langan *et al.* (1955), Rose *et al.* (1941) and Brown (1959) applied to the alcohol-ether extract did not vary significantly between themselves, but the value obtained with the colorimetric reaction of Abell *et al.* (1952) applied to this extract was significantly lower.

It was subsequently found that the alcohol-ether extraction procedure failed to extract about 5 p.c. of the serum cholesterol. This was established by determination of the serum cholesterol level with the method of Abell *et al.* (1952), in which the cholesteryl esters are saponified by addition of alkali to serum, and by application of the colorimetric reaction of Abell *et al.* (1952) to the non-saponifiable matter prepared in the manner described in this paper. It is nevertheless clear that the method of Zlatkis *et al.* (1953), as modified by MacIntyre and Ralston (1954), overestimated the level of cholesterol in serum. A detailed description of the magnitude of the errors in this method is given elsewhere (Brown, 1961a).

Cholesteryl stearate.

The colorimetric reactions of Zlatkis *et al.* (1953), Zak *et al.* (1954) and Pearson *et al.* (1953), as described by Leppänen (1956), were applied to amounts of cholesteryl stearate sufficient to give optical densities in the range of 0·08 to 0·10. The molecular extinction coefficients were respectively 13 p.c., 20 p.c. and 21 p.c. lower than those obtained for free cholesterol. In the original method of Abell *et al.* (1952) the colorimetric reagent was applied to lipids after saponification, but it is of interest that cholesteryl stearate was underestimated by 60 p.c. when the colorimetric reaction of Abell *et al.* (1952) was

TABLE 1.

Serum cholesterol values in 10 sera obtained by a number of different techniques.

	Serum	Alcohol-ether extract				Non-saponifiable extract				Digitonide
Colorimetric reaction	Zlatkis *et al.*	Durrum *et al.*	Rose *et al.*	Abell *et al.*	Brown	Durrum *et al.*	Rose *et al.*	Abell *et al.*	Brown	Abell *et al.*
	346	285	323	287	275	287	312	285	283	288
	224	168	174	155	171	147	148	150	153	153
	253	241	241	223	228	215	218	210	213	216
	312	298	271	256	258	256	255	245	243	247
	358	271	291	259	268	238	253	243	241	248
	212	231	246	198	225	189	187	198	190	205
	258	271	273	237	272	252	262	235	246	237
	340	295	290	257	276	264	264	256	258	258
	337	240	242	225	231	226	222	226	229	225
	293	205	216	191	209	202	199	194	194	189
Mean (mg. p.c.)	293	250	257	229	246	228	232	224	223	226
σ_i (mg. p.c.)	2·1	4·4	5·2	6·8	3·2	8·7	5·4	2·8	6·3	6·2

The colorimetric reactions were applied to the serum and its extracts as indicated in the table and the text.

applied to a dry film of 0·2 mg. of the ester. The underestimation of the cholesteryl stearate by several of the methods used appeared to be due to its insolubility in colorimetric reagents containing acetic anhydride, acetic acid and sulphuric acid. The colorimetric reactions of Rose *et al.* (1941), Trinder (1952), Brown (1959) and of Zlatkis *et al.* (1953), after its modification by Langan *et al.* (1955), gave identical molecular extinction coefficients for ester and free cholesterol when these were present in amounts sufficient to give optical densities greater than 1.

DISCUSSION.

The most troublesome problems associated with the choice of a standard procedure for determination of serum cholesterol were stated by Bloor (1916, 1943) who noted that while most workers regard the digitonide procedure as the most accurate method "the effect of alkali in saponification and the effect of other compounds than cholesterol being precipitated are objections to complete faith in it". Rapid oxidative changes in cholesterol by the combined action of heat, air and alkali have in fact been amply demonstrated (Bergström and Wintersteiner, 1941). However, autoxidation of cholesterol need not affect its determination with Liebermann-Burchard reagent (Norcia, 1958). In fact, for the purposes of its determination with digitonin and Liebermann-Burchard reagent, cholesterol is reasonably stable during the conditions of saponification

reported in this paper (Brown, 1961b). Furthermore, the adoption of a system of internal standards in this work balances changes in serum cholesterol during saponification against changes in standard cholesterol and compensates for any errors due to the additional difficulty mentioned by Bloor (1916) and Boyd (1935) of completely extracting cholesterol from an alkaline phase with solvents.* It is concluded that the low values obtained with the digitonide procedure reported in this paper are due to the greater accuracy of this method.

It is known that a method suitable for the determination of cholesterol in human serum may not be applicable to extravascular tissues such as skin with its mixture of sterols, slow and fast acting with Liebermann-Burchard reagent (Moore and Bauman, 1942). The various colorimetric reagents also vary among themselves in their specificity. The Tschugaeff reaction is said to be specific for 3β-hydroxysteroids and Liebermann-Burchard positive sterols have been found in faeces which were Tschugaeff negative (Danielsson and Gustafsson, 1959). Furthermore, methods accurate for human serum may be inaccurate when applied to other sera. Thus the Tschugaeff and Liebermann-Burchard reactions applied to the non-saponifiable matter of human serum gave similar values for cholesterol in the work reported in this paper, but gave widely divergent values for the cholesterol in a similar extract prepared from monkey sera (Blohm, Kariya and Laughlin, 1959). Liebermann-Burchard positive sterols other than cholesterol have also been reported to occur in dog sera (Anderson, 1927). Autoxidized products of cholesterol may also react differently with the various colorimetric reagents for cholesterol and some of these substances have been found in human serum (Prelog, Ruzicka and Stein, 1943), although even after saponification Abell *et al.* (1952) found by a countercurrent distribution technique less than 1 p.c. of the autoxidized products of serum cholesterol soluble in petrol-ether. For these reasons the remarks in this paper apply more particularly to accurate determination of cholesterol in human serum, and the term "cholesterol" is used in its conventional clinical sense of Liebermann-Burchard positive matter which can form an insoluble digitonide.

Non-saponifiable extract. Colorimetric determination of cholesterol in the non-saponifiable extract is complicated by brown substances formed during saponification with ethanolic alkali (Bloor, 1915; Ireland, 1941; Sperry and Brand, 1943) and it is possible this was one reason why Abell *et al.* (1952) used mild conditions for saponification. Gardner and Gainsborough (1934) found that impurities in the alcohol were responsible and that they could be eliminated by unspecified methods. Other workers have minimized the interference by using internal standards (Bloor, 1915), certain salts (Csonka, 1916)

* The hydrolysate was acidified prior to extraction by Boyd (1935) and by Sperry and Brand (1943). In general, colorimetric determination of the cholesterol in this extract may be complicated by the serum fatty acids present though Sperry and Brand (1943) obtained accurate values using the Liebermann-Burchard reagent without chloroform.

or wet extraction techniques (Sperry and Brand, 1943). Purification of the ethanol with m-phenylenediamine hydrochloride (Trinder, 1952) is more effective than the alkaline reflux procedure of Man and Gildea (1932) and it is used routinely in this laboratory. Acetone promotes the development of the brown colour. Aqueous alkali cannot be used as it does not completely hydrolyse cholesteryl esters (Thaysen, 1914; Fex, 1920).

Other substances may interfere with the colorimetric determination of serum cholesterol in the non-saponifiable extract. Carotene develops colours with the Liebermann-Burchard reagent (Levine and Bieri, 1934) and Kinley and Krause (1958) believe that Vitamin A in the non-saponifiable extract reacts with the colorimetric reagent of Zlatkis *et al.* (1953). The practical significance of these observations is unknown. On the other hand, Gardner and Gainsborough (1934) found only trivial amounts of substances interfering with determination of cholesterol in the non-saponifiable extract when the Liebermann-Burchard reagent was used. In fact, a number of colorimetric reactions applied to this extract give values similar to those obtained after purification of the cholesterol with digitonin (Abell *et al.*, 1952; Trinder, 1952; Hanel and Dam, 1955; Brown, 1959; Morris, 1960; Wright *et al.*, 1960). The data in this and a subsequent paper (Brown, 1961a) confirm this view.

The most widely used method for preparing the non-saponifiable extract is due to Abell *et al.* (1952). However, neither the procedure for saponification nor the Liebermann-Burchard reagent is completely effective with cholesteryl stearate. The difficulties of saponifying cholesteryl stearate are described elsewhere (Brown, 1961b) but its concentration in human serum is low (Klein, 1960) and the values for serum cholesterol were not significantly altered when more vigorous saponification was used in the method of Abell *et al.* (1952) (unpublished data). On the other hand, as much as 75 p.c. of the acids esterified to the cholesterol of animal tissues may be stearic and palmitic acids (Swell, Law, Field and Treadwell, 1960) and it is advisable that more vigorous conditions be used for saponification of the cholesteryl esters of animal tissues than were recommended by Schoenheimer and Sperry (1934) and Abell *et al.* (1952) for human serum. In the method of Brown (1959) saponification is complete even though cholesteryl stearate is the only ester present. Moreover, the colorimetric reaction used in this method overcomes the lack of colour stability and sensitivity associated with the Liebermann-Burchard reaction, while the colour developed has similar spectral characteristics to that produced by the Tschugaeff reaction so that it is possible this method and that of Hanel and Dam (1955) have similar specificities for cholesterol.

Alcohol-ether extract. The most-used method for serum cholesterol determination over the last 40 years is that of Bloor (1916). In this the Liebermann-Burchard reaction is carried out in a solution in chloroform of the dry residue of an alcohol-ether extract of serum. That the influence of this method is still widely felt may be seen in the recommendation by King and Wootton (1951)

of the procedure of Sackett (1925), which is essentially the procedure of Bloor (1916), and in the publications of Wright *et al.* (1960); Keys, Fidanza, Scardi, Bergami, and di Lorenzo (1954); Van Handel and Zilversmit (1957). The procedure of Sackett (1925) was also used by MacIntyre and Ralston (1954) to verify the accuracy of their version of the method of Zlatkis *et al.* (1953) before the recommendation of the latter method by King and Wootton (1956).

A number of early workers (Mueller, 1916; Denis, 1917; Bloor, 1917; Luden, 1917; Myers and Wardell, 1918) obtained high estimates with the procedure of Bloor (1916). Values for serum cholesterol obtained with this method and expressed as percent in excess of those obtained after saponification and precipitation of the cholesterol with digitonin are given in the following list together with the references: 15 p.c., Yasuda (1931); 17 p.c., Schoenheimer and Sperry (1934); 10 p.c., Reinhold (1936); 17 p.c., Nyons (1938); 6 p.c., Keys *et al.* (1954); 16 p.c., Anderson and Keys (1956); 4 p.c., Hood and Angervall (1957); 8 p.c., Wright *et al.* (1960); 7 p.c., Morris (1960). The error in the method of Bloor (1916) is due to the cholesteryl esters according to Sperry and Brand (1943) and to Saifer and Kammerer (1946). Thus the optical density expressed as percent in excess of that developed with the equivalent amounts of cholesterol has been variously recorded for the palmitate ester as 20 p.c. Reinhold (1936), 30 p.c. Kelsey (1939), 30 p.c. Sperry and Brand (1943), 25 p.c. Yasuda (1936); for the stearate ester 25 p.c. Yasuda (1936) and 13 p.c. Kenny (1952); for the oleate ester 13 p.c. Kenny (1952) and 12 p.c. Carr and Drekter (1956); and for the isolated cholesteryl esters of calf plasma 25 p.c. Teeri (1944) and pig serum 23 p.c. Yasuda (1936). Usually more than 70 p.c. of human serum cholesterol is esterified and an error of about 15 p.c. in the procedure of Bloor (1916) seems well established. The errors using plasma are more difficult to assess. Oxalate, but not heparin, may reduce the values for cholesterol and other lipids by as much as 15 p.c. owing to osmotic and other effects on the blood cells (Kirk, Page and Van Slyke, 1934; Schmidt, 1935; Sperry and Schoenheimer, 1935; Kenny, 1952). Pronounced reduction in the ratio of ester to free cholesterol will affect total cholesterol determination with the method of Bloor (1916) but the normal variation in the ratio has only a small effect (Kenny, 1952).

There are many modifications of the Liebermann-Burchard reaction. Chloroform was omitted by Schoenheimer and Sperry (1934), Sperry and Brand (1943) and Abell *et al.* (1952). The data in this paper show that rather accurate values are obtained when the reagent is applied to the dry residue from an alcohol-ether extract. However, the cholesteryl esters with long-chain saturated acids may be underestimated by this approach and the apparently accurate values might be due to a favourable combination of errors. Nevertheless, Carr and Drekter (1956) dissolved the esters in very small quantities of chloroform before the addition of the Liebermann-Burchard reagent and their data together with that of Cramer and Isaksson (1959) show that certain cholesteryl esters can be accurately determined in the presence of appropriate quan-

tities of chloroform. Cramer and Isaksson (1959) applied the reagent to dry residue from an alcohol-ether extract and obtained cholesterol estimates 4·6 p.c. greater than those given by the digitonide method of Sperry and Webb (1950).

The data in this paper show that high estimates are obtained when the colorimetric reactions of Rose *et al.* (1941) and Brown (1959) are applied to the dry residue from the alcohol-ether extract. Cholesteryl stearate and oleate are accurately determined but the polyunsaturated esters are overestimated (Brown, 1959). Rhodes (1959), in fact, proposed a method for the determination of polyunsaturated acids in serum based on the extraneous colour that they develop with the colorimetric reagent of Zlatkis *et al.* (1953) for cholesterol determination. In addition, the data in the present paper show that the long-chain saturated-esters of cholesterol may be underestimated by the colorimetric reactions of Zlatkis *et al.* (1953) and Zak *et al.* (1954).

Both accurate and high values for serum cholesterol have been claimed by various workers using one or other of a variety of colorimetric reactions modified from that of Zlatkis *et al.* (1953) and applied to an unpurified solvent extract of serum. Thus Klungsöyr, Haukenas and Closs (1958) obtained values 14 p.c. higher than those obtained with the method of Schoenheimer and Sperry (1934), while the data given in this paper show that the modified method of Langan *et al.* (1955) gave high estimates when applied to the dry alcohol-ether extract of serum. However, it is widely believed that the method of Zak *et al.* (1954) is accurate. A further variation of the method of Zlatkis *et al.* (1953) was introduced by Chiamori and Henry (1959) who used a mixture of ferric chloride and acetic acid to precipitate the serum proteins before colour development with sulphuric acid. The values obtained were similar to those obtained after determination of the cholesterol in the non-saponifiable extract which would appear to confirm the accuracy of the method. However, in the absence of appropriate standardization, the varying accuracy obtained with all these modified methods could be held to be due to incomplete extraction of cholesterol or its esters from the serum proteins, incomplete colour development with the saturated esters, to extraneous colour development, or to a combination of these factors.

In general, the standardization of methods omitting saponification should be further investigated with serum cholesteryl esters isolated, for example, by chromatographic methods (e.g. Brown, 1959) and with tests on the recovery of total cholesterol when its extraction from the serum proteins is carried out prior to colour development. An example of the latter test is given in this paper when the recovery of cholesterol in the non-saponifiable extract prepared according to Abell *et al.* (1952) was taken as being 100 p.c., and the recovery of cholesterol in the alcohol-ether extract was found to be about 5 p.c. less. It might also be noted at this juncture that the use of cholesteryl acetate to standardize methods for cholesterol determination is unsatisfactory as it shows neither the effects of the solubility nor the unsaturation of the physiological esters of cholesterol. It will also be seen that the direct colorimetric determina-

tion of the serum cholesteryl esters after paper chromatography is not as accurrate as considered by Hansen and Dam (1957) or by Quaife, Geyer and Bolliger (1959).

Clarity on these points is very desirable as, apart from any other considerations, the procedures associated with saponification cannot be simply adapted to inexpensive automatic analytical techniques.

Direct serum cholesterol determination. Ferro and Ham (1960), Pearson *et al.* (1953) and Zlatkis *et al.* (1953) added colorimetric reagents directly to serum. It is interesting to note that Ferro and Ham (1960) added Liebermann-Burchard reagent without chloroform to serum and obtained values 15 p.c. higher than those obtained after determination on the non-saponifiable matter.

According to Carr and Drekter (1956) the method of Pearson *et al.* (1953) gave values 10 p.c. lower than those obtained with the method of Schoenheimer and Sperry (1934), but according to Baerts *et al.* (1956) the two methods give values agreeing to within a few percent. According to Leppänen (1955) the method of Pearson *et al.* (1953) gave similar values to that of Bloor, Pelkham and Allen (1922), the latter method giving values similar to those obtained with the Liebermann-Burchard reagent applied to the non-saponifiable matter (Bloor, 1928). Josephson and Gyellenswand (1957), on the contrary, believe the method of Pearson *et al.* (1953) gives high values. When compared with the digitonide procedure of Schoenheimer and Sperry (1934), and methods essentially the same as that of Abell *et al.* (1952), values high by approximately 30 mg. p.c. were in fact obtained by Morris (1960) and by Best *et al.* (1954). Wright *et al.* (1960) obtained values about 16 p.c. too high both for the method of Pearson *et al.* (1953) and several modified versions of it. The reasons for these high values are unknown. In uraemic plasma unidentified substances may increase the values by as much as 96 mg. p.c. (Baerts *et al.*, 1956). The Pearson method underestimates cholesteryl stearate (this paper) but not cholesteryl palmitate, according to Wright *et al.* (1960).

The method of Zlatkis *et al.* (1953) has been widely used. Its authors obtained similar estimates with the method of Schoenheimer and Sperry (1934) and this has led to much confusion. In the hands of Morris (1960) purification of the acetic acid used (MacIntyre and Ralston, 1954) reduced the mean values for serum cholesterol by more than 100 mg. p.c. below those obtained with unpurified acid and in this paper all other references to the direct procedure of Zlatkis *et al.* (1953) refer only to work in which the purified acetic acid was used.

When the colorimetric reagent used by Zlatkis *et al.* (1953) was applied to the non-saponifiable matter of rabbit serum, the values obtained were 21 p.c. lower (range 15-25 p.c.) than those obtained when the reagent was applied to the same serum samples (Herrmann, 1957). When the colorimetric reagent was applied to an alcohol-ether extract of rabbit serum the values obtained were also lower than those obtained with the original method (Day and Peters, 1958)

but the colour differed from that developed with the preparation of standard cholesterol (Schwartz, C. W., private communication). A similar difference in colour was seen in two of the ten human sera reported in this paper, but it was not associated with the highest values for cholesterol. The reason for the discoloration is unknown, but a number of steroids give various colours with the reagent (Bischoff and Turner, 1958).

The method of Zlatkis *et al.* (1953) is sensitive to bromide (2 mEq./l) encountered clinically in serum (Rice and Lukascewicz, 1957). Bromide can pass into the alcohol-ether extract and interfere with colorimetric determination at this stage. Langan *et al.* (1955) obtained high estimates with the method of Zlatkis *et al.* (1953) and human serum. In uraemic plasma the estimates were about 96 mg. p.c. too high but in the absence of uraemia they were too high by 38 mg. p.c. (σ_1, 3·1 mg. p.c.) (Baerts *et al.*, 1956) or 36 mg. p.c. (σ_1, 22 mg. p.c.) (Brown, 1961a).

The conclusion to be drawn from the data presented in this paper is that, in general, purification of serum cholesterol is the most important factor in its accurate determination, and that the preparation of the non-saponifiable extract is an important stage in the procedure for purification. As early as 1917 Luden demonstrated that elimination of the interfering substances of icteric plasma by the procedures associated with saponification reduced errors found in the method of Bloor (1916). The accuracy of more recently-developed methods in which saponification is omitted requires further investigation. The omission of saponification is usually justified on the grounds of simplicity (Bloor, 1916); Denis, 1917; Andes, 1934; Saifer and Kammerer, 1946; Pearson *et al.*, 1953; Zak and Ressler, 1955; Hood and Angervall, 1957). Nevertheless, the non-saponifiable extract was prepared very simply by Abell *et al.* (1952) and although the temperature and the time of saponification may be altered to advantage, the method should at present form the basis for serum cholesterol determination in both research and clinical work.

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A FILTRATION PROCEDURE FOR DETERMINING SERUM CHOLESTEROL WITH DIGITONIN

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SUMMARY.

A precise and accurate method was developed for the determination of cholesterol with Liebermann-Burchard reagent after its isolation as the digitonide. The cholesteryl digitonide was isolated by filtration instead of by the usual procedure of repeated centrifugation and decantation of wash fluids. The method is as precise as methods in which colorimetric reactions are applied to the non-saponifiable extract and in which accuracy is obtained with the preparation of internal standards. Parameters are given for the maximum amounts of cholesteryl esters which may be saponified at 37° C. over a 30-minute period. The difficulties of saponifying cholesteryl stearate due to its insolubility are discussed. It is shown that a protracted period of saponification at 65° C. need not affect the accuracy of determination of cholesterol with digitonin.

INTRODUCTION.

Although they are technically difficult to use, methods based on precipitation of cholesterol with digitonin have been widely used for checking the accuracy of less difficult methods for serum cholesterol determination (Andes, 1934; Saifer and Kammerer, 1946; Steiner, 1949). The methods in which digitonin is used are liable to errors due to loss of cholesteryl digitonide during the repeated decantation of wash fluids, and by adherence of cholesteryl digitonide to the glass rod used to break up the precipitate after centrifugation (Sperry, 1938; Ireland, 1941; Sperry and Webb, 1950; Hawk, Oser and Summerson, 1954; Weigensberg and MacMillan, 1959). The use of filtration instead of centrifugation might appear to resolve these problems but the filtration procedures so far developed (Windaus, 1910; Mueller, 1916; Okey, 1930; Man and Peters, 1933; Kirk, Page and Van Slyke, 1934; Boyd, 1938) are no longer used for serum cholesterol determination. In the experience of the writer and others (Mueller, 1916; Webster, Nichols and Chaikoff, 1959) the principal difficulty encountered during filtration is caking of the cholesteryl digitonide on the filter. This difficulty has been overcome with the method reported in this paper.

Opinions differ widely on the manner in which saponification of cholesteryl esters introduces errors into determination of total serum cholesterol with digitonin. Thus Kirk *et al.* (1934) and Bloor (1916, 1943) believed that the conditions necessary for complete saponification of the esters without destruction of

cholesterol were critical yet Schoenheimer and Sperry (1934) saponified the esters under quite different conditions of time and temperature from those used by Kirk *et al.* (1934). In fact a great variety of conditions have been used by such well-known workers as Man and Peters (1933), Sere, Chaikoff and Dauben (1948), Abell, Levy, Brodie and Kendall (1952) and Sperry (1955).

The effect of the various concentrations of alkali used on the rate of saponification is also obscure. Indeed, Nyons and Polano (1940) believed sodium ethylate was required for complete saponification of the esters yet Boyd (1936) claimed that alcoholic potash was satisfactory. This problem is further complicated by the use of ethanol or an ethanol:acetone mixture as solvent during saponification.

The present study was undertaken to clarify the effect of saponification on the precipitation of cholesteryl digitonide and the effect of the solvent on the ease of saponification of various cholesteryl esters. As a result of the work a method was developed for the determination of serum cholesterol based on isolation of the cholesteryl digitonide by filtration. The application of the method for checking the accuracy of less complicated methods for determination of serum cholesterol has been reported elsewhere (Brown, 1959, 1961a, 1961b). A description of a method for determining the ratio of free to total cholesterol in human serum in which the filtration procedure is used is given in the appendix of this paper.

Materials and Methods.

Powdered glass BDH was washed with hot chromic acid until no visible reduction of the acid occurred overnight at 110° C. After thorough washing with water on a Buchner funnel the powder was dried at 110° C. and shaken with Liebermann-Burchard reagent for one hour. It was then successively washed with water, alcohol, ether and dried by suction. This washing procedure was also used to recover the glass powder after use.

Long-handled steel spoon to hold *ca.* 30 mg. of glass powder (Fig. 1).

Filter columns (Argon Instruments, Sydney). Each filter column holds 10 ml. of fluid above a sintered glass disc (porosity 4, 1·1 cm. diameter, 1·0 mm. thick) set immediately above a stem 5 mm. in diameter. The dimensions of the disc are critical for easy operation of the method.

Rack to hold filter columns (Fig. 2). Two rows of 12 clips each are rivetted to a frame made for insertion into a water bath of dimensions 40 cm. x 20 cm. x 15 cm. The clips are 1 cm. in diameter, spaced 3 cm. apart along each row.

Q and Q glass taps cat. no. SC1/3. Fourteen ungreased taps are connected to a manifold distributing industrial dry nitrogen. Screw clips may be used in place of the taps.

Very flexible clear plastic tubing (Moulded Products). It is used to connect the filter columns to the manifold taps.

Filter stem caps. Two centimetre lengths of the plastic tubing are stoppered at one end with glass rods. They are used to cap the filter stems as indicated in Fig. 1.

Pumpett (Shandon Co., London). It is used with a 5 ml. bulb pipette with an orifice which has been narrowed in a Bunsen flame.

Certified thermometer, 0-100° C.

Cholesterol and the acetyl, oleayl and stearyl esters, BDH reagents.

Digitonin, BDH reagent. A 0·5 p.c. solution in 50 p.c. ethanol (Sperry and Webb, 1950).

Ethyl ether, anaesthetic grade (Woolwich Elliott). It was dried over calcium chloride and distilled before use.

Acetone, AnalaR.

Absolute ethanol, CSR; purified over m-phenylenediamine hydrochloride (Trinder, 1952).

Potassium hydroxide, AnalaR. The solution used for saponification was prepared according to the instructions of Schoenheimer and Sperry (1934) which were subsequently adopted by Sperry and Brand (1943), Sperry and Webb (1950), Abell *et al.* (1952) and Anderson and Keys (1956). A 33 *p.c. solution of potassium hydroxide* is prepared by adding 50 gm. of pellets to 100 ml. of water. 2·0 *p.c. ethanolic potassium hydroxide* is prepared by making 6 ml. of the 33 p.c. aqueous solution to 100 ml. with ethanol.

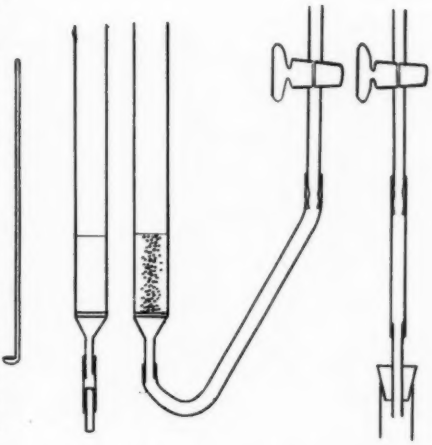

Fig. 1. Left to right: steel spoon; filter column with capped stem; nitrogen passing up through the filter; the connection between the nitrogen manifold and the filter column for forcing out the Liebermann-Burchard reagent.

Liebermann-Burchard reagent (Abell *et al.,* 1952). Thoroughly mix 10 vol. of acetic anhydride (Merck AR), at a temperature below 10° C., with 1 vol. of sulphuric acid (AnalaR) and bring the mixture to the temperature of the water bath (20° C. or 26° C., see text) just before use. Commence use of mixture within one hour of preparation.

FILTRATION TECHNIQUE.

Isolation of cholesteryl digitonide.

A filter column is connected to the second tap on the manifold with the very flexible clear plastic tubing (Fig. 2). The filter column is mounted in a clamp and nitrogen is slowly passed up through the filter and 2 ml. of 0·5 p.c. digitonin solution are added. Three ml. of 1:1 ethanol-acetone mixture containing 0·13-1·0 mg. of cholesterol are pipetted directly into the digitonin solution. Allow the nitrogen gas to mix the solutions for about 30 seconds. With the gas flow continuing disconnect the plastic tubing and immediately cap the filter stem with the caps described above. Add *ca.* 30 mg. of glass powder to the mixture. The powder sediments very rapidly while the cholesteryl digitonide remains suspended for some time. The filter column is tapped sharply to pack the glass powder over the sintered disc. The filter is fixed upright in a rack, covered with an inverted plastic cone stopper to reduce evaporation and left overnight to allow complete precipitation of the cholesteryl digitonide (Sperry and Webb, 1950). Next morning the mother liquor is swirled gently around the meniscus to loosen any material adhering to the glass while the cholesteryl digitonide remains situated as a soft layer above the glass powder. A further 30 mg. of glass powder are added to the mother liquor and the filter column is tapped sharply to facilitate its penetration into this layer of cholesteryl digitonide. The mother liquor is drawn through the filter by suction (water pump) or allowed to drain away while other matters receive attention. Immediately the meniscus has entered the digitonide layer, the suction line is disconnected, 5 ml. of acetone are washed down the walls of the filter column and

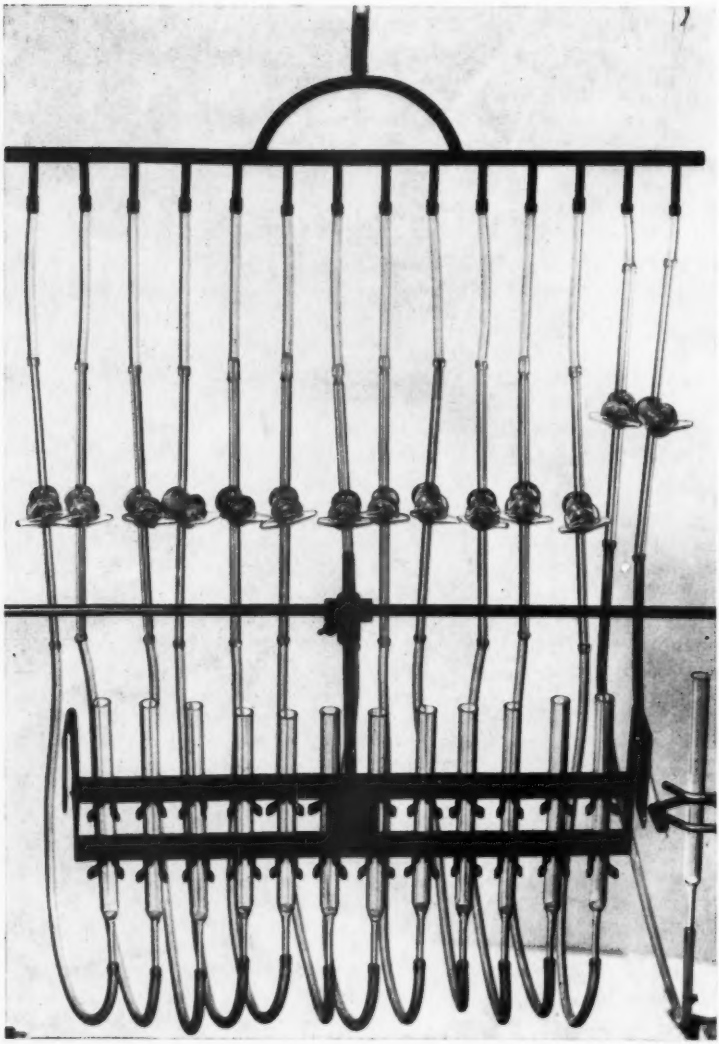

Fig. 2. The filter column and manifold assembly. The rack and filter columns with their attached plastic tubing are situated in a water bath during colour development.

the suction line is reconnected. It is disconnected again as the meniscus of the acetone enters the digitonide layer. The filter stem is capped and 5 ml. of distilled, anaesthetic ether are very forcibly injected into the digitonide and glass layers to completely disrupt them into a suspension. Tap the filter columns sharply to facilitate packing of the glass powder. The jet of ether is easily controlled with a Pumpett and a 5 ml. bulb pipette with a fine orifice. The last 0·5 ml. of ether should be directed around the upper (inside) wall of the column to avoid terminal splashing of the digitonide suspension on to the wall of the column.

Allow 30 minutes for the cholesteryl digitonide to flocculate. Add a further 30 mg. of glass powder and remove the cap. Draw out the ether or allow it to run out under gravity. Dry the digitonide by sucking air briskly through the filter for one minute. The cholesteryl digitonide now exists as a firm, compact disc over the glass powder. With the exception of this terminal operation, the gauge on the water pump should show a pressure greater than 10 mm. of mercury during all suction operations.

Colorimetric determination of cholesteryl digitonide.

The water bath containing its rack is adjusted to $20 \pm 0{\cdot}1°$ C. The filter column is tapped to break the cholesteryl digitonide layer and it is then connected to the manifold via the plastic tubing with the taps turned off; it is then placed in the rack and its position there recorded.

Liebermann-Burchard reagent (5 ml.) at 20° C. is then washed slowly down the wall of the filter column. After the addition of about the first ml. of reagent, a stream of nitrogen is passed up through the filter until the digitonide has dissolved in the 5 ml. of reagent. This takes from one to three minutes depending on the amount of digitonide present. During the latter part of this period the filter column is removed from the rack and the contents are swirled around to ensure complete solution of the digitonide, which should be verified by inspection. It is then replaced in its position in the rack in the water bath and the appropriate manifold tap turned off. With a series of filter columns in the rack the colour reagent is added at three-minute intervals. After 55 minutes each filter is removed from the water bath, its outer surface is dried and it is disconnected from the manifold. It is mounted on a retort stand, a nitrogen line from the manifold is connected to the top of the filter column (Figs. 1 and 2) and some reagent is blown through the filter into a 1 cm. cuvette. This is used to wash the cuvette and it is discarded. Further reagent is blown into the cuvette and its absorbance is measured at 620 mμ between 58 and 63 minutes after beginning the colour development. During this time the absorbance should not vary by more than 1 p.c.

RESULTS.

Precision and accuracy of the method.

The standard deviation of individual values in a series of 24 determinations of cholesterol was 1·2 p.c. of the mean value. Beer's law was obeyed with the regression line passing through the origin. This relation should be checked when setting up the method. Twelve replicate determinations of cholesterol in the presence of an equal amount of cholesteryl acetate showed a mean recovery of 100 p.c. with a variance not significantly different from that of pure cholesterol determination.

A simplified method for colorimetric determination.

The method described was designed to minimize the influence of room temperature during the whole time of colour development. However, the

following simplified method may be used for cholesteryl digitonide determination without materially affecting the results:—

The filter column containing the dry cholesteryl digitonide is clamped upright outside the water bath and the Liebermann-Burchard reagent (5 ml.) at 20° C. introduced as before. With the nitrogen flowing, disconnect the plastic tubing and immediately cap the filter stem. The contents of the filter column remain above the sintered disc and the filter column may be used like a test tube. Place the capped filter column in the water bath at 20° C. and after 55 minutes dry the column, remove the cap, and blow out the colour reagent as already described. When only a few filters are being used the temperature of the water bath may be raised to 26° C. and the colour read at 30-33 minutes.

The effect of saponification on the determination of cholesterol as the digitonide compound.

Cholesterol (10 mg. p.c.) in 2·0 p.c. ethanolic potash was kept at 65° C. The concentration of cholesterol was measured with the digitonide procedure before incubation commenced and at half-hour intervals thereafter for a period of three hours. No change was found in the ability of the cholesterol to form the digitonide compound and to develop colour with the Liebermann-Burchard reagent. The same result was obtained using an ethanol:acetone mixture in place of the ethanol despite the very heavy discoloration of the hot solvent mixture that occurred in the presence of potassium hydroxide.

The amount of cholesteryl ester split by mild saponification.

The acetyl, oleayl and stearyl esters of cholesterol were each dissolved in 9·4 ml. of ethanol or ethanol:acetone at 60° C. and then allowed to precipitate at room temperature. 0·6 ml. of 33 p.c. potassium hydroxide was added and the flasks were continuously shaken for 30 minutes in a water bath at 37° C. The alkali was neutralized and the free cholesterol was determined. The difference between the recovery of free cholesterol under these conditions and after saponification of identical preparations at 65° C. for one hour was found. Further checks were made on the largest amounts of ester examined in each series by extending the period of saponification to two hours at 65° C. The significance

TABLE 1.

Maximum amount of ester (expressed as mg. p.c. of cholesterol) saponified at 37° C. for 30 minutes in 2 p.c. alcoholic KOH.

	Cholesteryl ester		
	Acetate	Oleate	Stearate
Ethanol	>500	120	5·0
Ethanol : acetone	>500	>500	44

of the differences was computed with the t-test for "one-sided" comparisons. The data showing the amount of cholesteryl ester saponified at 37° C. were selected and expressed as the content of cholesterol, rather than the ester, per 100 ml. of solution. These data are shown in Table 1.

It is evident that the stearyl ester is relatively difficult to saponify at 37° C. It is also of interest that the precision of the values for free cholesterol obtained with the filtration and saponification procedures combined was 1·7 p.c. of the mean value when expressed as standard deviation of the individual values.

Solubility of the cholesteryl esters.

The data in Table 2 show the solubility of several cholesteryl esters in ethanol and ethanol:acetone at 37° C. Saturated solutions of the esters at 60° C. were prepared and shaken continuously at 37° C. for about 18 hours. They were centrifuged at 37° C. and the concentration of the ester in the supernatant fluid at this temperature was determined gravimetrically.

TABLE 2.

Solubility of cholesteryl ester at 37° C. tabulated as gm. of cholesterol per 100 ml.

	Cholesteryl ester		
	Acetate	Oleate	Stearate
Ethanol	1·9	0·56	0·13
Ethanol:acetone	3·4	1·2	0·17

Concentration of alkali and saponification of cholesteryl stearate.

The differences in rates of saponification of cholesteryl esters shown in Table 1 do not appear to be simply due to differences in solubility, although the relative insolubility of cholesteryl stearate probably makes it the most difficult to saponify of all the quantitatively important cholesteryl esters of human serum. The effect of concentration of alkali on saponification of cholesteryl stearate is therefore of particular interest in the determination of conditions for complete saponification of serum cholesteryl esters generally. For this reason, in judging the importance of concentration of alkali on saponification of serum cholesteryl esters, cholesteryl stearate was taken in amounts equal to that which could be just completely saponified at 37° C. for 30 minutes in 2·0 p.c. potash (0·36N) in ethanol or ethanol:acetone (Table 1) and the effect of varying the normality of the potash under these standard conditions was then tested (Table 3). It will be seen that the rate of saponification can be affected considerably by the concentration of alkali used for the purpose. Review of the data in Tables 1, 2 and 3 suggests, moreover, that the way in which saponification of a mixture of cholesteryl esters is affected by variation in concentration of alkali depends greatly on the composition of the mixture of esters.

TABLE 3.

The effect of concentration of alkali on hydrolysis of cholesteryl stearate.

Normality of KOH	Percentage hydrolysis in	
	ethanol	ethanol : acetone
0·36	100	100
0·30	98	95
0·24	91	88
0·18	80	70
0·12	61	52
0·06	34	28

DISCUSSION.

The filtration procedure described in this paper for isolation of cholesteryl digitonide is more easily controlled than the usual procedures based on the centrifugation and decantation of wash liquors as described by Schoenheimer and Sperry (1934) and Sperry and Webb (1950). The cholesteryl digitonide is isolated in a comparatively short time since it takes 1½ hours to wash and dry 12 samples. The use of the contentious stirring rod (Sperry and Webb, 1950) to break up lumps of precipitate after centrifugation is avoided, while the precipitate is so finely suspended during the ether wash that time should be allowed for flocculation to occur before removal of ether through the filter. In these circumstances, it may be assumed that the washing of the cholesteryl digitonide is particularly efficient. It has been shown elsewhere (Brown, 1959, 1961a, 1961b) that the precision obtained during determination of serum cholesterol levels with the filtration procedure is about the same as that obtained with colorimetric determination of serum cholesterol in the dry non-saponifiable extract. However, the procedure suffers from the usual limitations of the Liebermann-Burchard reaction. The time and temperature of colour development must be strictly controlled to allow prediction of the time of maximum colour development. A certified thermometer should be used on this account. Anderson and Keys (1956) believe reagent purity affects rate of colour development, which is in agreement with experience in this laboratory.

For best results the time for maximum colour development should be verified with each set of determinations, as is also recommended by Anderson and Keys (1956). Digitonide procedures are tedious to use and for reasons given elsewhere (Brown, 1961a, 1961b) they need only be used in special circumstances for determination of total serum cholesterol. They are more useful in measuring the ratio of free to esterified cholesterol and various 3β-hydroxysteroids in addition to cholesterol.

The data reported in this paper verify the opinion that the mixed cholesteryl esters of serum are readily saponified in a 1:1 mixture of alcohol and acetone.

On the other hand, the rate of saponification of the long-chain saturated esters in alcohol alone is reduced, and more vigorous conditions for saponification must be used to saponify the same amount of esters in a given time. Contrary to Bloor (1916, 1943) the data justify the general assumption that the method of Schoenheimer and Sperry (1934) can be used as a standard procedure against which the accuracy of less elaborate procedures may be measured, provided the precautions taken by Sperry and Webb (1950) are observed.

It should also be pointed out that the values given in this paper for the amount of cholesteryl esters saponified under specified conditions of solvent, time and temperature must be related to the way in which the solutions were prepared for saponification. Saponification was induced in saturated solutions of cholesteryl ester in the presence of undissolved ester. The values given approach the maximum rate at which the various esters are saponified. In methods for cholesterol determination generally, alcoholic alkali may be applied to a dry film of the residue from a solvent extract of serum or other tissue and saponification is then allowed to proceed without agitation of the solution. In these circumstances the rate of solution of the ester may appreciably limit the rate of saponification. Thus only 66 p.c. of cholesteryl stearate was hydrolysed when 5 ml. of 2 p.c. potassium hydroxide in ethanol were applied to a dry thick film of 0·1 mg. of the ester and saponification was carried out at 37° C. for 55 minutes without agitation. Recalculation of data in Table 1 shows that at least 0·4 mg. of cholesteryl stearate can be saponified in 30 minutes at 37° C. if the ester is already largely in solution at the start of saponification. Whether as a dry thick film of ester or already in solution at the start of saponification, 0·4 mg. of cholesteryl stearate is saponified in less than 10 minutes by 5 ml. of 2 p.c. potassium hydroxide in ethanol at 65° C. without agitation (unpublished data).

Saponification of serum cholesteryl esters at 37° C. for 30 minutes was introduced by Schoenheimer and Sperry (1934) to minimize changes in the cholesterol by alkali and air. The earlier practice of concentrating the alkali to dryness during saponification at higher temperatures than that used by Schoenheimer and Sperry (1934) may well have led to errors in serum cholesterol determination (Man and Peters, 1933; Yasuda, 1931). However, the data given in this paper show that a wide range of conditions may be used for saponification in a constant volume of solvent without necessarily affecting the accuracy of the determination of cholesterol. This is a practical matter as the purity of the cholesterol recovered in the non-saponifiable matter may depend on the degree of saponification of other lipids in the serum which are relatively difficult to saponify. It was for this reason that in the method for determination of serum cholesterol with perchloric acid (Brown, 1959) saponification was carried out at 65° C. for one hour even though the cholesteryl esters were saponified in less than half of this time.

Acknowledgment. This work was supported by a grant from the National Health and Medical Research Council.

APPENDIX.

Determination of the ratio of free to total cholesterol in human serum using digitonin precipitation of 3β-hydroxysteroids.

Free cholesterol. Inject 40 ml. of 1:1 ethanol:acetone mixture into 2 ml. of serum contained in a 50 ml. volumetric flask. Bring the solution to 65° C. in a water bath, stopper the flask firmly and maintain it at this temperature for 30 minutes with occasional shaking. Allow the solution to cool to room temperature and make it up to 50 ml. with the solvent mixture. Evaporate the solvent from 40 ml. of the solution and dissolve the residue in 10 ml. of fresh solvent mixture. Precipitate the free cholesterol in 3 ml. of this concentrated extract by the method described in this paper.

Total cholesterol. Take 3 ml. of the concentrated extract of serum lipids, add 0·2 ml. of 33 p.c. potassium hydroxide, mix and saponify at 65° C. for 20 minutes. Cool the solution and neutralize it with glacial acetic acid to a few drops in excess of a phenolphthalein end point. Make the solution up to 10 ml. with 1:1 ethanol-acetone and mix it well before taking 3 ml. for precipitation with digitonin.

Standard cholesterol. Take 3 ml. of a 50 mg. p.c. solution of cholesterol in 1:1 ethanol:acetone mixture, add 0·2 ml. of 33 p.c. potassium hydroxide and then follow the instructions given for determination of total cholesterol.

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THE INFLUENCE OF ANTI-INFLAMMATORY CORTICOSTEROIDS ON THE GROWTH OF THE CHICK EMBRYO AND THE MANIFESTATIONS OF THE SIMONSEN PHENOMENON

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SUMMARY.

When a suitable dose of prednisolone is given to the 12-day chick embryo two hours prior to the CAM inoculation of adult fowl leucocytes the focal count produced is significantly reduced. Inoculation of prednisolone 12 hours after leucocytes has no significant effect.

In vitro treatment of leucocytes with prednisolone in dosages effective in the egg has no cytotoxic effect on the leucocytes.

Much larger doses of prednisolone are needed to inhibit the splenic enlargement caused by intravenous inoculation of adult leucocytes.

When 12-day embryos are treated with prednisolone given by the natural air space, growth is inhibited and with larger doses oedema and retardation of feather growth are seen. Intravenous inoculation of the drug inhibits growth, but the pathological changes do not occur.

A wide range of anti-inflammatory steroids has been tested for their action in reducing focal counts on the CAM and in inhibiting embryo growth.

The activities found differ significantly from the results obtained in experimental mammals and from clinical experience. The unduly high activity of 11 β hydroxy derivatives is noted and discussed.

INTRODUCTION.

The reaction produced by grafting adult fowl tissue or cells on the chorioallantois was noted by Murphy (1916) and was first studied in detail by Dantchakoff (1916). More recently Simonsen (1957) produced splenomegaly in young chickens by injecting 18-day embryos intravenously with adult fowl leucocytes. He characterised the effect as a graft-versus-host reaction. Burnet and Boyer (1960) obtained a similar type of reaction by inoculating adult fowl leucocytes on to the chorioallantoic membrane (CAM) of 12-day chick embryos. After four days' incubation easily visible opaque foci were present on the membranes. The numbers of foci showed a wide scatter but with adequate numbers of embryos the median values showed a linear dose/effect relationship. In the course of this work it was noted that hydrocortisone added to the inoculum (a)

reduced the number of foci, (b) slowed down the rate of growth of the embryo between the 12th and 16th days. A preliminary report on these effects of corticosteroids has already appeared (Burnet and Warner, 1960).

The effect of cortisone on the growth of the chick embryo was described by Karnofsky *et al.* (1951), Sames and Leathem (1951), and Evans (1953). They showed an inhibition of growth rate with doses of 0·3 mg. per embryo and Karnofsky also noted that hydrocortisone was more active in this respect than cortisone. Tests of cortisone on a graft-versus-host reaction in mice have not been reported but Billingham, Krohn and Medawar (1951) have shown that appropriate treatment with cortisone will prolong the survival time of skin homografts by a factor of 3 to 4. Their experiments indicated that the effect of cortisone depends more on its power to reduce a systemic development of the response rather than to weaken the local fulfilment of the immune response.

In the present paper we report more fully on the two responses to corticosteroids in the chick embryo. The action of prednisolone phosphate is examined in some detail and it is concluded that the effect on the foci on the CAM involves both the initiation of the reaction and the development of the host response to the initiated focus. The comparative activity of a fairly large series of anti-inflammatory corticosteroids is also examined on this system and incidentally a number of observations are recorded on the "corticosteroid syndrome" in the chick embryo.

Materials and Methods.

Chick embryos: Fertile eggs (White leghorn-Australorp hybrids) were obtained from a commercial source, and were used on the twelfth day of incubation.

Donor fowls: White leghorn and New Hampshire fowls from 6-12 months old were bled by either wing vein puncture or cardiac puncture, one-tenth volume of heparin (1000 i.u. BDH) being used as anticoagulant. After centrifugation for 10 minutes at 1000 r.p.m., the leucocytic layer was taken up into glass capillaries and spun for 5 minutes at 1000 r.p.m. The upper few millimetres containing most of the leucocytes were made up to approximately the original volume of blood in Dulbecco and Vogt balanced salt solution pH 7·4 containing 100 i.u. of penicillin and 50 i.u. of streptomycin per ml.

Corticosteroids: We are grateful to Glaxo Laboratories, England, for kindly providing us, through Dr. T. F. McCrae, with most of the drugs used. Solutions or suspensions of the drugs were prepared in antibiotic saline. For studies on the dose-effect relationship a ½ log 10 spacing of doses was employed. 0·1 ml. volumes of steroid dilutions were inoculated through a stabbed needle hole in the shell, into the natural air space, the egg being held vertical with air space uppermost for two hours at 38° C. before the CAM was dropped. Preliminary experiments had shown that absorption of the drug was as effective by this route as any other and it was preferred as introducing no complication for any subsequent manipulation of the embryo.

Experimental procedures: The CAM was dropped by the method of Burnet and Boyer (1960) and inoculated with 0·1 ml. of a suspension of adult fowl leucocytes prepared as described and usually containing $4\text{-}8 \times 10^6$ leucocytes per ml. Intravenous inoculations were performed in the manner described by Beveridge and Burnet (1946).

After four days' further incubation at 38° C., the embryos were removed, roughly dried of external moisture and weighed. For dry weight determinations, the embryos were dried in a vacuum oven at 80° C. for about eight hours.

The dropped area of the CAM was harvested, and the number of specific foci counted.

For the experiments in which intravenous inoculations of leucocytes were used, eggs were incubated for 5 days before embryos and spleens were harvested and weighed.

RESULTS.

The general character of corticosteroid action on the embryos.

There is a general uniformity of effect of the different anti-inflammatory corticosteroids that have been tested although the effective dose range varied widely from one to another. With an adequate dose the most evident effect when the embryos were examined after four days was a retardation of growth shown by reduction in weight and length of the embryos in comparison with untreated controls.

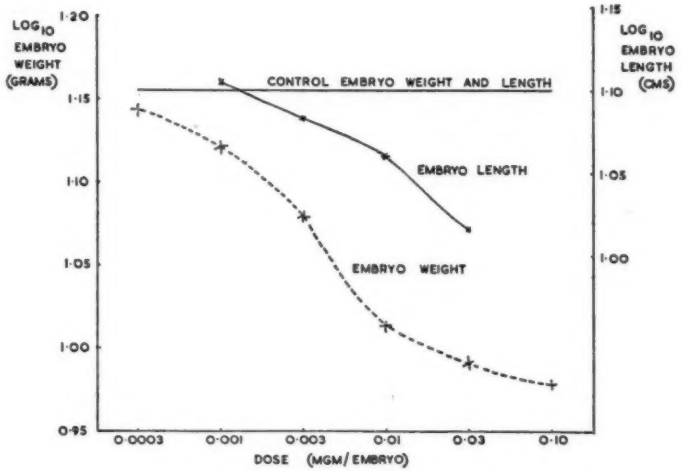

Fig. 1. Dose-effect relationship of prednisolone on chick embryo weight and length.

Fig. 1 shows the type of dose-effect relationship given by prednisolone. The curve has the usual sigmoid shape but with increasing doses irregularities appear as the toxicity of the drug becomes evident. There is an increasing mortality of the embryos and survivors may show oedema and anomalies of feather development. The accumulation of water is very apparent in the tissues under the neck and over the abdomen, retardation of feather growth is most evident on the back of the head. The water-retaining effect varies amongst the corti-

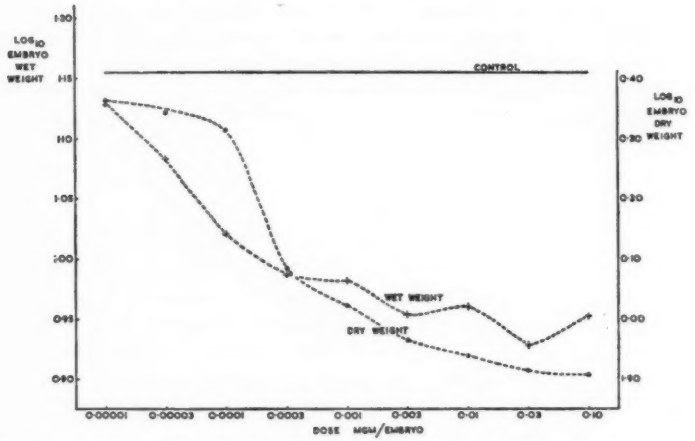

Fig. 2. Dose-effect relationship of 9 α fluoro 16 β methyl prednisolone on chick embryo wet and dry weights.

costeroids; the effect produced by 9 α fluoro 16 β methyl prednisolone phosphate is shown in Fig. 2 which compares the wet and dry weights. There are clearly advantages in using the dry weight if a complete picture of the growth inhibiting effect over the whole range is desired. The wet weights, however, give quite satisfactory results in the medium dose range and have been used for most of the work reported.

The action of prednisolone phosphate.

Effect on the embryo. Limited observations were made on the weights of liver and the three lymphoid organs of the chick embryo, spleen, thymus, and bursa of Fabricius. All organs weighed less than controls but while the ratio of liver weight to embryo weight was somewhat higher in controls and the spleen ratios about equal, both thymus and bursa showed a reduction to about half the control ratio.

TABLE 1.

Effect of prednisolone on chick embryo when administered intravenously and into the natural air space.

	Embryo weight (gm.)	No. of deaths	Cortisone syndrome
Control	13·9	1/24	0/23
0·1 mg. prednisolone into natural air space	10·4	14/39	22/25
0·1 mg. prednisolone intravenously	8·7	2/30	0/28

Embryos were harvested four days after inoculation of 0·1 mg. of prednisolone per embryo by either route. The last two columns respectively show the number of dead embryos when harvested and the numbers of live embryos showing the typical "cortisone syndrome".

The route by which prednisolone is administered has a significant influence on the results. Embryos inoculated intravenously show retardation of growth but the toxic effects as measured by mortality and the incidence of oedema and feather anomalies are much less than those seen when the drug is given by the air space route (Table 1).

Effect on focal lesions on the CAM. When a standard suspension of fowl leucocytes in 0·1 ml. is inoculated on the CAM of commercially raised embryos, the count of foci shows a relatively wide scatter with a few extreme values. In experiments on the effect of prednisolone and other corticosteroids on the focal count it was found best to use the median value using 10-20 embryos for each point. In order to indicate the nature of the experimental material used, Table 2 gives the actual focal counts recorded in the experiment on which the curve for prednisolone given in Fig. 3 is based.

There is a significant reduction in number of foci with 0·01 mg. per embryo and further reduction with increasing dose. The morphology of the foci is also affected. Lesions after doses from 0·03 mg. per embryo upwards are smaller,

TABLE 2.

Action of prednisolone on focal count: typical protocol.

Control	130, 110, 65, 62, 37, 37, 34, 29, (29) 27, 22, 18, 15, 14, 7, 5, 4
0·001 mg./embryo	111, 94, 79, 42, 38, 35, (29) 27, 26, 20, 12, 5, 3
0·003 mg./embryo	65, 55, 50, 44, 35, 30, (24) 19, 15, 14, 12, 5, 0
0·01 mg./embryo	42, 32, 19, 15, 15, (11) 7, 7, 6, 0, 0
0·03 mg./embryo	36, 18, 15, 10, 9, 6, (5) 5, 3, 3, 2, 1, 0
0·10 mg./embryo	16, 8, 3, 1, 0, (0) 0, 0, 0, 0, 0

The values shown are the actual counts of specific foci on membranes given 0·1 ml. of a standard suspension of adult fowl leucocytes on the CAM, and the indicated dose of prednisolone in 0·1 ml. into the natural air space. Median values are indicated in brackets.

TABLE 3.

Effect of route of administration of prednisolone upon the reduction of focal count.

Dose and mode of administration	Median focal count	
	Test	Control
0·05 mg. prednisolone into air space leucocyte suspension on CAM	2	18
0·05 mg. prednisolone mixed with leucocyte suspension on CAM	2	18
0·10 mg. prednisolone intravenously 2 hours before leucocyte inoculation on CAM	9	40
0·10 mg. prednisolone intravenously 22 hours before leucocyte inoculation on CAM	4	45

The test series were given the treatment indicated and for each experiment a control group was inoculated with leucocytes alone.

flatter and more sharply demarcated than in control membranes. The histological appearance suggests a lessened proliferative activity of the host cells (see Burnet and Boyer, 1961).

The route of administration of prednisolone has little influence on the results. Table 3 shows that the drug mixed with the leucocytes before inoculation has no greater effect than when given into the air space, while intravenous injection is also effective at any time up to 22 hours before inoculation of leucocytes on the CAM. Using the air space route it appears that at least two hours should elapse before leucocyte inoculation if an adequate effect is to be obtained. A longer period of 16-24 hours gave a greater effect in two experiments and all tests in which steroids were given various intervals after the leucocytes showed reduced or absent effect.

If leucocytes were treated *in vitro* with 0·03 mg. prednisolone per 0·1 ml. for 7 hours at 37° C., i.e. with the same total dosage of drug as was effective when given previously by the air space route, no reduction in count below the control was observed. A concentration of 10 mg./ml. was required to have a significant effect. Table 4 shows the relevant data for these experiments.

TABLE 4.

Effect of in vitro *treatment of leucocytes with prednisolone prior to their inoculation on the CAM.*

	Median focal count
Control leucocytes	16
Control leucocytes 7 hours at 37° C.	7
Leucocytes with 0·01 mg. PSL/ml. 7 hours	6
Leucocytes with 0·3 mg. PSL/ml. 7 hours	9
Control leucocytes 6 hours	22
Leucocytes with 1·0 mg. PSL/ml. 6 hours	17
Leucocytes with 10·0 mg. PSL/ml. 6 hours	5

Effect on splenic enlargement. When 12-day embryos are inoculated intravenously with 0·1 ml. of a standard leucocyte suspension the spleen weights five days later show a relatively wide scatter of values.

In our experiments on the effect of prednisolone, groups of 12-18 embryos have been used for each dose and the mean spleen weight per 10 gm. of embryo determined after five days. A dose of 0·1 mg. of prednisolone per embryo given intravenously either 5 hours before, together with or 4, 8, 12 or 24 hours after intravenous inoculation of leucocytes, has no effect in reducing the resulting splenomegaly. A dose of 0·75 to 1·0 mg. is required to produce a significant reduction of spleen weight (Table 5).

Burnet and Boyer (1960) have shown that the enlarged spleen following intravenous inoculation of adult leucocytes contained cells capable of producing

TABLE 5.

Effect of prednisolone on splenomegaly.

Mode of administration	Mean spleen wt./10 gm. embryo wt.	
	Test	Control
0·1 mg. prednisolone 5 hours before leucocytes	45 mg.	50 mg.
0·1 mg. prednisolone mixed with leucocytes	37 mg.	32 mg.
0·1 mg. prednisolone 8 hours after leucocytes	51 mg.	53 mg.
0·25 mg. prednisolone mixed with leucocytes	28 mg.	34 mg.
0·75 mg. prednisolone mixed with leucocytes	15 mg.	34 mg.
1·0 mg. prednisolone mixed with leucocytes	17 mg.	59 mg.
Normal 17 day embryos	—	6 mg.

Groups each contained from 12-18 embryos and were harvested 5 days after intravenous inoculation with leucocytes with or without prednisolone.

foci when transformed to further CAMs. The effective cells have been assumed to be descendants of the donor leucocytes, although this has not yet been adequately demonstrated. It was of interest therefore to see whether the number of such immunologically competent cells has been modified significantly by prednisolone treatment. Using Burnet and Boyer's technique the results shown in Table 6 were obtained.

From the median focal count and the number of cells inoculated, the number of cells per focus has been calculated. Despite the reduction of spleen size to less than one-third there is no reduction in the proportion of "competent" cells present.

TABLE 6.

Content of competent cells in spleens from prednisolone-treated and control spleens.

Inoculum	Mean spleen wt. at 5 days	Mean spleen wt./10 gm. embryo weight	Cells per focus from passage of spleens
Control leucocytes intravenous	76 mg.	52 mg.	5×10^3
1·0 mg. prednisolone with leucocytes intravenous	22 mg.	24 mg.	8×10^3

Spleen weights were obtained five days after inoculation. Dispersed cell suspensions were prepared from the 5 day spleens and titrated on 12 day CAMs.

Action of different corticosteroids on embryo weight and focal count.

An extensive series of quantitative experiments on the effect of graded doses of various corticosteroids on embryo weight and focal count has been carried out. The results are shown in a series of dose-effect curves (Figs. 3 to 8).

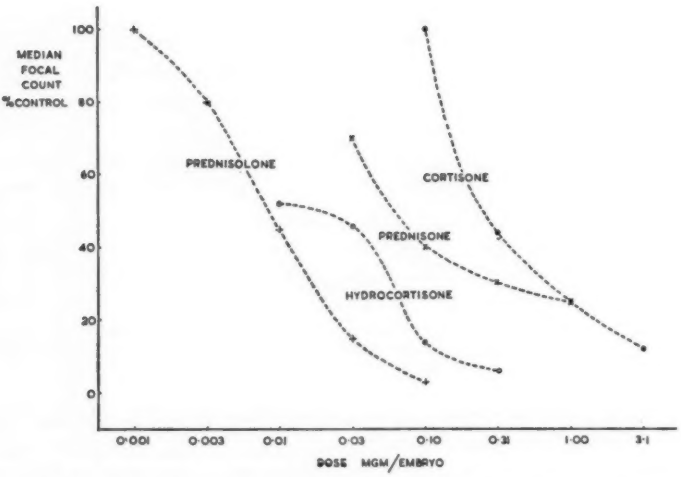

Fig. 3. Influence of four corticosteroids on focal count on the chorioallantoic membrane. This shows the advantage of possessing:

(1) Δ^1 groups: Cortisone < prednisone
Hydrocortisone < prednisolone

(2) 11 β hydroxy groups: Cortisone < hydrocortisone
Prednisone < prednisolone

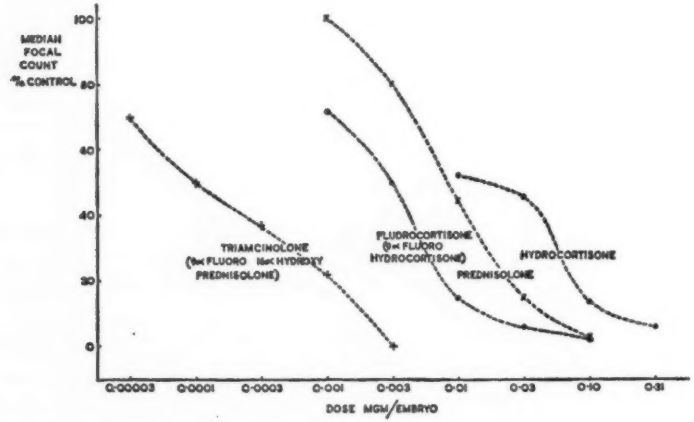

Fig. 4. Influence of four corticosteroids of focal count on the chorioallantoic membrane. These graphs show the increased activity conferred by the presence of:

(1) 9 α fluoro groups: Hydrocortisone < fludrocortisone.

(2) 16 α hydroxy groups: to assess the activity of this group, triamcinolone is compared with prednisolone by allowing for a tenfold increase in activity of the latter for the 9 α fluoro groups. Contrary to mammalian tests, the 16 α hydroxy group is seen to further increase activity.

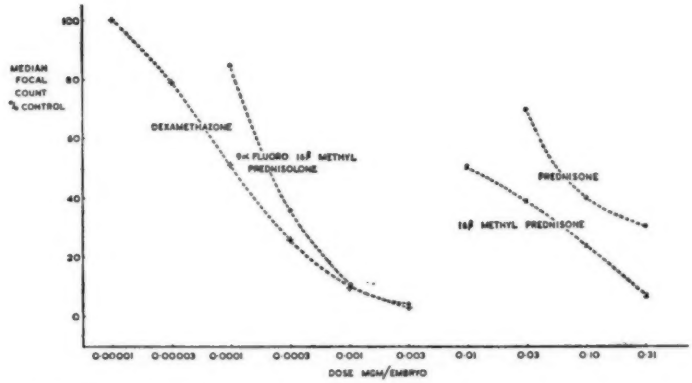

Fig. 5. Influence of four corticosteroids on focal count on the chorioallantoic membrane. The activity of the 16 β methyl group is compared with its parent compound and its α isomer.
(1) Prednisone $<$ 16 β methyl prednisone.
(2) 9 α fluoro 16 β methyl prednisolone only slightly less active than dexamethazone.

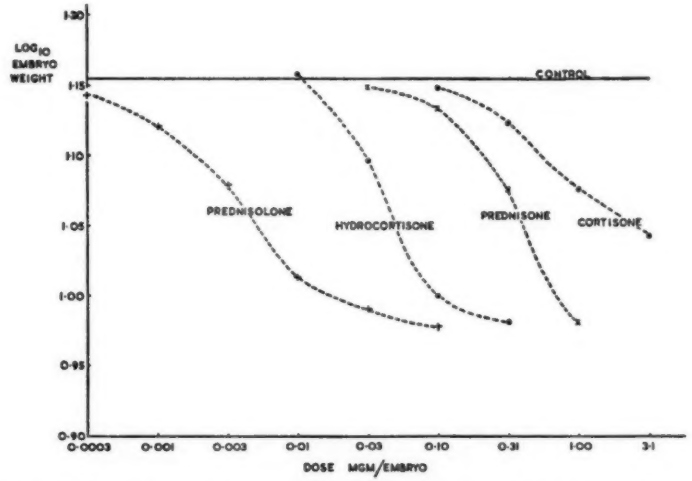

Fig. 6. Influence of four corticosteroids on chick embryo weight. The pattern seen here closely follows that of Fig. 3, i.e. the Δ^1 groups, and particularly the 11 β hydroxy groups, are both responsible for an increase in activity.

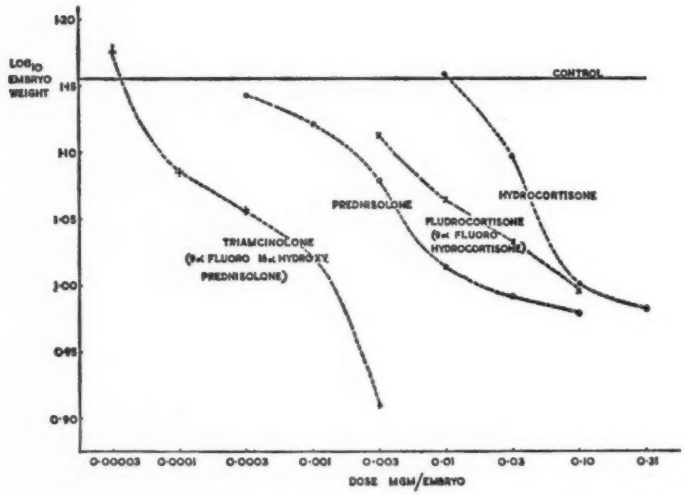

Fig. 7. Influence of four corticosteroids on chick embryo weight. By applying the same conditions as in Fig. 4, both the 9 α fluoro and the 16 α hydroxy groups cause an increase in activity. The positions of fludrocortisone and prednisolone, however, reversed in comparison with Fig. 4.

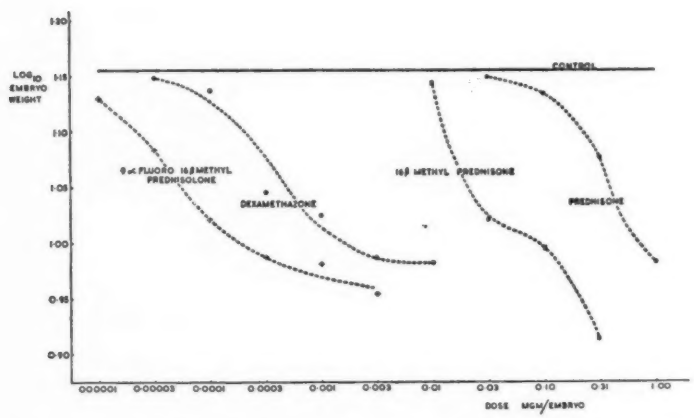

Fig. 8. Influence of four corticosteroids on chick embryo weight. The 16 β methyl group is again seen to increase activity, but the 16 α isomer is the more active isomer as determined by this test.

In all these experiments, the standard technique of air space inoculation of a suspension or solution of corticosteroid two hours before CAM inoculation of leucocytes was employed. By comparing the actual dose required to reduce the focal count to 25 p.c. of the control, and the embryo weight by three grams, the relative activity of some of the compounds was determined and is shown in Table 7.

TABLE 7.

Relative activity of corticosteroids as judged by standard mammalian tests and the two chick embryo methods.

Compound	Standard biological activity	Order of activity	Activity from focal count	Order of activity	Activity from embryo weight	Order of activity
Cortisone	0·8	1	0·1-0·2	1	0·03	1
Hydrocortisone	1·0	2	1·0	4	1·0	3
Prednisone	3-4	3	0·3	2	0·1	2
Prednisolone	4-5	4	3	5	10	6
Triamcinolone	5-6	5	80	7	125	7
16 β methyl prednisone	6-7	6	0·6	3	2·5	4
Fludrocortisone	10	7	9	6	3	5
Dexamethazone	30	8	210	9	125	7
9 α fluoro 16 β methyl prednisolone	30	9	125	8	800	9

The agents are arranged in the accepted order of clinical and biological activity. This order is not the same as those given by the chick embryo test. All activities shown are in terms of that of hydrocortisone which is given a value of 1·0.

DISCUSSION.

The growth inhibiting effect of cortisone on the chick embryo was first shown by Karnofsky *et al.* (1951), who indicated that the most severe effect appeared to be initiated on the 8th day. They concluded that this was perhaps due to its inhibiting the formation of a new function, tissue, or substrate essential for growth. More recently, however, Pickman *et al.* (1959), giving cortisone to the chick embryo on the initial day of incubation, have shown a weight inhibition on the fourth day.

In general, our experiments indicate that prednisolone given by the air sac route produces essentially similar effects to those described by Karnofsky *et al.* (1951) for cortisone. The conspicuous features are inhibition of growth, oedema, anomalies of feather growth and diminished viability. The most interesting aspect of our results is probably the dissociation of growth inhibition from the other pathological effects on the chick embryo when the prednisolone is given as a single intravenous injection. The oedema, etc., are thus not necessarily related to the process responsible for growth inhibition (perhaps an effect on the pituitary). Possible hypotheses are that for their production the drug must be available in the circulation for a long period at low concentration or

that they are due to the action of a derivative produced by enzymic action as the prednisolone passes through tissues before being distributed by the circulation. We have not the facilities for the elaborate chemical work that would be needed to analyse the situation further.

The development of focal lesions on the chorioallantoic membrane following inoculation of adult fowl leucocytes has been described by Burnet and Boyer (1960), who postulate that each focal lesion arises as a result of the interaction of a mature immunologically competent cell with a component of the CAM carrying one or more antigenic determinants not present in the donor. The lesion develops as a result of multiplication of both donor and host cells with secondary inflammatory and reparative changes. It is assumed that by interaction of the immunologically active donor cells and their descendants with antigen on or liberated by host cells pharmacologically active agents are liberated with irritating and growth proliferating effects. The greater part of the lesion is clearly of host cell origin and Burnet and Boyer's identification of certain pyroninophil cells as descendants of the initiating donor cell is still only tentative.

The effect of prednisolone and the other anti-inflammatory corticosteroids in inhibiting the development of these foci could be based hypothetically on one or more of the following:

(a) a cytotoxic effect on the donor leucocytes;

(b) an inhibition of the normal liberation of growth-stimulating agents following immunological contact between donor and host cells;

(c) an inhibition of many types of growth response in the embryo host, including those stimulated by such agents.

The possibility of a direct action on the leucocytes can be eliminated since prednisolone mixed with leucocytes before inoculation has actually less effect than the same amount given by air space inoculation some hours before the leucocytes were inoculated.

The foci on membranes of prednisolone-treated eggs are smaller as well as fewer in number. There is undoubtedly some diminution in the host response, and in the present state of knowledge it seems probable that the findings can be based on two well-recognised effects of the anti-inflammatory corticosteroids.

1. They weaken all types of cellular responses to immunological stimulation.
2. They inhibit, presumably by central action, all types of proliferative processes in the chick embryo.

To initiate a focus a cell must lodge in such a situation that it can both make contact with a foreign antigen and find the appropriate conditions that will allow initial division both of itself and of adjacent host cells. The variability of replicate focal counts indicates that accidental factors can play a significant part and it would therefore be expected that a general weakening of the contact

responses would diminish the probability that a given cell would initiate a focus. Once the process has been initiated the effect of corticosteroid can only be in diminishing the size of the focus by reducing the intensity of the host response.

We do not wish to elaborate on the part played by the donor cells and descendants until a means of identifying them unequivocally is available. We would indicate, however, that if Burnet and Boyer's identification of the morphological appearance of these cells is correct, sections of foci inhibited by prednisolone frequently show a much higher proportion of "donor" to host cells than in untreated lesions. In addition, the result given in Table 6 shows that there is no evident failure of competent cells to multiply in the spleen in the presence of prednisolone. Much more work needs to be done on the numbers, distribution and immunological variability of donor cell descendants but provisionally we believe that the effect of the active corticosteroids can be summed up as reducing the likelihood of effective initiation of a lesion on the CAM and when the lesion has been initiated diminishing the cellular host response without much effect on the continued proliferation of descendant cells of donor origin.

The assay of corticosteroids in the chick embryo has been described by Karnofsky (1954) and Stock *et al.* (1951) who studied the effect of many different corticosteroids on the chick embryo weight. Karnofsky established that the activities of the adrenal corticosteroids as assayed in the chick embryo do not parallel the results found in mammals and that the chick embryo is specially sensitive to hydrocortisone. Our own work has been confined to those corticosteroids of current interest for clinical use as anti-rheumatic agents. In general, the results are consistent with Karnofsky's conclusions.

The substitution of a β hydroxy group for the keto group at 11, as shown by comparison of hydrocortisone with cortisone or prednisolone with prednisone, greatly increases activity as judged either by the effect on embryo weight or in inhibiting CAM foci. The possibility that the chick embryo at the stage used in our experiments lacks the enzymic equipment to reduce the keto form will obviously need consideration in comparing our results with those obtained in experiments on mammals or from clinical experience. Examination of Table 7 will show a number of other indications of differences in relative activity when tests made by the two chick embryo methods are compared with the standard clinical tests.

Glenn (1959) has suggested that variation in the rates at which corticosteroids are metabolised is important in accounting for differences in biological activity. This seems likely to be a major factor in our own results.

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THE EFFECT OF COLLOIDAL SILICA ON BLOOD COAGULATION

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SUMMARY.

Colloidal silica accelerates blood coagulation by adsorption and partial denaturation of a specific plasma protein, the Hageman factor. Quantitative measurements show that the activity of the silica depends on the particle diameter. It is suggested that geometrical relations between the silica and the protein molecules account for these differences in activity. The implications of certain anomalies of this reaction are considered.

INTRODUCTION.

The surface of silica is very active in the initiation of blood coagulation and of certain other related changes (Margolis, 1957, 1958a, 1959a). Although we have now a fairly coherent picture of these reactions in biological terms (Hardisty and Margolis, 1959; Margolis, 1959a, 1960a) very little is known about their physical aspects. In the course of previous work many different forms of silica were used. Quartz, tridymite, cristobalite, vitreous silica and kaolin were all excellent activating materials. They were used as granules, powders or suspensions and it was consistently found that the activity increased with the state of division of the solid, obviously owing to the increase of the specific surface area ($cm.^2/g.$). The most finely divided and therefore the most active preparations tested at the time were commercial silica aerogels and colloidal dispersions of 10-20 mμ particle diameter. But this trend did not continue with further diminution of the particle size. Silica sols made by neutralising dilute sodium silicate with HCl were almost totally inert in spite of their even smaller particle size (about 2 mμ) and hence proportionately greater surface area. Therefore somewhere between 10 and 2 mμ there is a critical minimum particle size, below which silica ceases to activate plasma (Margolis, 1959b).

Application of adequate methods for the preparation of well-specified silica dispersions and of measuring their clot-promoting ability made it possible to study quantitatively these relations. This investigation, a preliminary account of which was presented elsewhere (Margolis, 1960b), was undertaken in the hope that the results may shed some light on the physical basis of the plasma/surface reactions.

Methods.

Vitreous silica.

Vitreous silica powders were prepared from ground silica tubing. The coarser particles were graded by passing through wire mesh sieves, retaining the 120-200 mesh fraction. They were then washed with three changes of deionised water and dried at 100° C. In order to exclude surface contamination by traces of adsorbed organic impurities, some samples were heated to 600° C. This treatment did not enhance their activity. Finer powders were graded by fractional sedimentation in deionised H_2O. The specific surface areas were estimated by adsorption of methylene blue (cf. Nevo *et al.*, 1955). A standard sample of glass microspherules was used as reference allowing for the difference in specific gravity between glass (2·8 in this case) and silica (2·2).

Colloidal silica.

The smallest particle sols were made by rapidly passing cooled solutions of 1-5 p.c. Na_2SiO_3 through a column of the cation exchange resin Zeo-Karb 225. The effluent (pH 3-4) was then stabilised at pH 8·5 by adding 2 p.c. ammonia. The colloidal particles thus prepared (2-3 mμ diam.) remain relatively stable for some days when kept at 4° C. Intermediate size particles (4-6 mμ) were prepared from the above by heating at 100° C. or autoclaving at 15 lb./inch pressure for 15-60 min. The initial pH was adjusted to various levels (5·5-8·5) and the resulting particle diameter was found to depend on pH, more alkaline suspensions yielding smaller particles in this range. Commercial preparations were used as a source of still larger particles (7-30 mμ). These were diluted with H_2O and also passed through the resin columns and adjusted to pH 8·5. The mean particle diameter was estimated by light scattering (courtesy of Mr. E. Elbing, Department of Physical Chemistry, University of Sydney), and in two cases (15 and 30 mμ) confirmed by electron microscopy (courtesy of Dr. H. Hoffman, C.S.I.R.O.). Since all these suspensions consist of fairly uniform spherical particles, the approximate surface area of each could be calculated from the particle diameter. As a corollary, the diameter of the irregular, ground particles of vitreous silica is therefore best expressed in terms of "equivalent spherical diameter" calculated from the known specific surface area. Immediately before use all

TABLE 1

Specifications of silica preparations

	Material	Surface area (m^2/g)	Equivalent spherical particle diameter*	Description
1	Vitreous silica	0·02	135 μ	Powder
2	Vitreous silica	0·07	39 μ	Powder
3	Vitreous silica	0·24	11·2 μ	Suspension
4	Vitreous silica	5·10	0·53 μ	Suspension
	Colloidal silica			
5	"Syton W20" (Monsanto)	90	30 mμ	Colloidal
6	"Ludox HS" (DuPont)	180	15 mμ	Colloidal
7	"Ludox SM" (Du Pont)	386	7 mμ	Colloidal
8	Heated silica sols (see text)	450-675	4-6 mμ	Colloidal
9	Unheated silica sols (see text)	900-1,350	2-3 mμ	Colloidal
10	Dried gels from 5-9	0·04 (external)	135 μ	Powder

* Diameter of spherical particles of equivalent surface area/g.

suspensions were adjusted to pH 7·5. The relevant physical specifications are set out in Table 1. Although the accuracy of these data is not of high order, the probable error being in the range of ± 20 p.c., this error is not significant when compared with the magnitude of the differences in biological activity of these preparations.

Dry gels.

Dry gels were obtained from the above colloidal dispersions by pouring them on to stainless steel trays and drying in a current of warm air. They were then crushed and treated as vitreous silica, retaining the 120-200 mesh fraction. The specific gravity of these gels was approximately one-half that of vitreous silica. For more detailed description of the properties of silica sols and gels the reader is referred to the monograph by Iller (1955).

Clot-promoting activity.

Clot-promoting activity was measured as follows. All surfaces with which blood or plasma came in contact were treated with silicone (M.S.200) diluted fifty times in petroleum ether. Fifty volumes of venous blood were mixed with one volume of 20 p.c. trisodium citrate and centrifuged at low speed to obtain platelet-rich plasma, and then at 2000*g* to obtain platelet-poor plasma. One part of platelet-rich plasma was frozen at − 20° C. for one hour, thawed and added to six parts of platelet-poor plasma. The mixture was used as the substrate in all the experiments. Silica suspensions (0·05 ml.) were delivered into a row of siliconed containers and mixed with 0·2 ml. of the substrate plasma. The mixtures were gently agitated for 2 min. at 37° C. and recalcified with 0·05 ml. of 0·1M $CaCl_2$ using an automatic delivery syringe. Conventional glass clotting tubes were found to be inade-

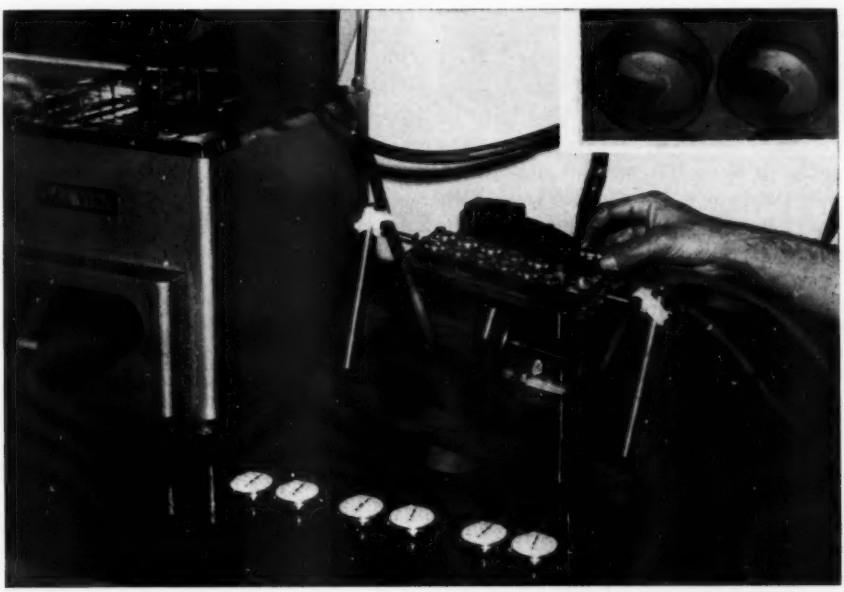


Fig. 1. Apparatus for accurate determination of clotting time. Inset shows crescents of plasma in the metal caps. See text.

quate for rapid and accurate work. The tests were therefore performed in disposable siliconed tin-plate caps ⅝ in. × ⅜ in. which were fitted into specially cast sockets in a metal warm stage heated by circulating water at 37° C. The whole stage was mechanically rocked 90 times/min. It was also inclined about 15° to horizontal, which produced a smooth to and fro flow of the contents in a crescentic fashion along one edge of each tray (Fig. 1). The clotting times were measured using a separate stop watch for each parallel sample. Up to ten tests could thus be performed simultaneously and an extensive series (50-70 tests) completed before any significant ageing of plasma took place.

Tests for plasma kinin.

Tests for plasma kinin were performed on isolated rat uterus (Margolis, 1958a). The procedure was as described for coagulation experiments except that the substrate plasma was not enriched with platelets, activation was carried out at room temperature and instead of adding $CaCl_2$, 0·1 ml. of the plasma + silica mixture was applied for one minute to a horn of rat uterus in oxygenated Ringer's solution in 10 ml. siliconed organ bath at 30° C.

RESULTS.

Activity of suspensions.

The clotting time of recalcified plasma is quantitatively related to the amount of activating surface. When the clotting time is plotted against the concentration of powdered silica on a log/log scale a linear relation is obtained within a wide range of values (Margolis, 1957). This may be used as a basis for quantitative comparisons.

With the method described, the clotting times are reproducible well within 5 p.c. on the same sample of plasma (Margolis, 1958b). The reproducibility from day to day is somewhat less, mainly owing to small technical variations in the collection of blood. The usual clotting time with the optimal amount of silica is 45-50 sec., while that of the blank (i.e. with 0·05 ml. of H_2O instead of the suspensions) about 300 sec. Even with unsatisfactory plasma samples (due to traumatic blood collection) the internal relations are well maintained within each experiment, although all the clotting times are proportionately shortened.

Fig. 2 shows the results of a typical experiment. The clotting times were plotted as a function of the weight (2A) or surface area (2B) of the added silica. As may be expected, weight for weight the smaller particles of powdered vitreous silica are more active than the larger ones (1A). In fact, the activity is almost a direct measure of the specific surface area which is inversely proportional to the particle diameter (2B). With colloidal particle dimensions this relation no longer holds. Below 30 mμ the smaller particles are less active even on a weight basis, but since the activity is a function of the surface area the data in Fig. 2B are more informative in this respect.

The smallest particles simply fail to activate the plasma. They do not inhibit the action of larger particles. When mixtures of the two are added to plasma together the only effect is one of dilution of the active by the inactive suspensions (see Fig. 6).

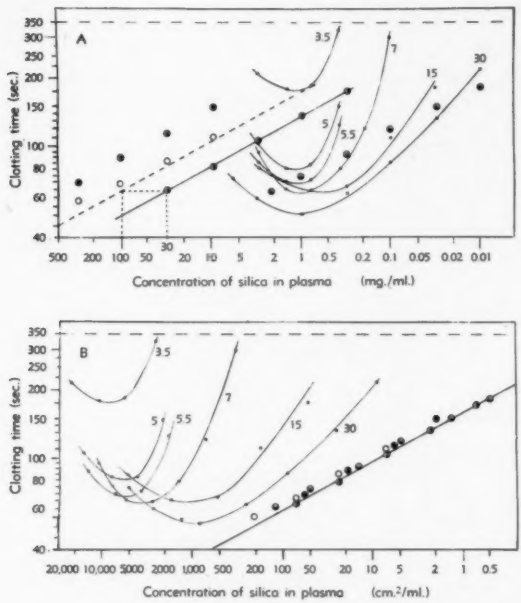


Fig. 2. Clotting time of recalcified plasma (see Methods) as a function of the concentration of silica in the plasma expressed as mg./ml. in (A) and as cm.²/ml. in (B). Equivalent spherical particle diameter of vitreous silica: (●) 135 μ; (○) 39 μ; (◑) 11·2 μ (reference standard); (◒) 0·53 μ. Colloidal silica (.): the figures beside the curves indicate the particle diameter in mμ. The method of calculating relative activity is illustrated by the interrupted lines in (A).

Two further features of the relations in Fig. 2 must be considered. Firstly, beyond a certain limit, a further increase of activating surface not only fails to shorten the clotting time, but has the opposite effect. This negative phase occurs also with powdered silica and kaolin (Margolis, 1960b) although it cannot be easily demonstrated with the larger particles because of the prohibitively large amounts which would be necessary (e.g. 5-10 gm. of the 120-200 mesh powder/ml. of plasma would be required). It is probable that the inhibition of clotting by excess surface is due to non-specific removal of one or more of the limiting factors in the chain of coagulation reactions.

The second and more interesting anomaly is the loss of linear relation between log concentration and log clotting time as the particle size decreases. Below a certain critical concentration of silica there is a rapid fading of activity with further dilution of the colloid. This anomaly will be discussed later, but in the meantime it introduces a complication in the measurements of activity which does not arise with the larger particles, when the relative activity (i.e.

activity per unit weight or surface area) remains constant throughout the relevant part of the curve. In the colloidal range, this value varies from point to point. It is therefore necessary to define some other constant as a basis for comparison. The absolute minimum clotting time could be used as such, but it is rather ill-defined and besides it cannot be measured with the coarser powders. Instead, the point of maximal relative activity was therefore chosen. It is located by drawing tangents to the curves in the direction parallel to the linear vitreous silica curves. For instance, the activity of the 3·5 mμ sample as compared with the standard powder of 11·2 μ equivalent spherical diameter is 30 p.c. on a weight basis (interrupted lines in Fig. 2A) or $\frac{30 \times 3{\cdot}5}{11{\cdot}2 \times 1000} = 0{\cdot}0094$ p.c. on a surface basis.

Using the same powder as a standard, repeated tests carried out on different days on the same samples of colloidal silica gave fairly reproducible values (co-efficient of variation ± 20 p.c.) considering the low slope and wide range of the curves.

The mean results of three separate estimations on those samples of silica which were best specified by physical measurements are shown in Fig. 3. Curve 1 represents the variation of activity/gm with the particle diameter. It may be seen that in the range 2-7 mμ small increases in the particle size produce very large increases in activity (e.g. hundredfold increase between 2·5 and 3·0 mμ). In fact, this relationship is probably a much more sensitive index of the particle size than are physical measurements. However, the limiting factor in the accuracy is not so much the assay of activity as the uniformity of the particle size and the intrinsic difficulty in physical measurements of small particles (cf. Iller, 1955). We must therefore allow for a possible error of some 0·5 mμ in the 2-5 mμ range. The activity per unit weight reaches its peak at about 40 mμ and then falls as the surface area decreases.

Curve 2 is derived from (1) and expresses the activity per unit surface area. Here there is no real maximum and the particles in the range 0·1-135 μ are approximately equally active.

Activity of dried gels.

Silica aerogels (Table 1), prepared as described, are composed of branching aggregates of the colloidal particles which still retain their individuality. The specific gravity of the gels was 1·0-1·2, i.e. about 50 p.c. that of the ultimate particles. This means that approximately half of the volume is occupied by pores. The pores are too small to allow any significant interchange of protein coagulation factors between the interior and exterior of the granules and hence only the external surface need be considered in the measurement of activity. Vitreous silica of the same particle size (120-200 mesh) was used as a standard.

The results in curve 3 (interrupted) in Fig. 3 are shown in terms of activity per unit area. As with the suspensions, the activity of the gels falls as the

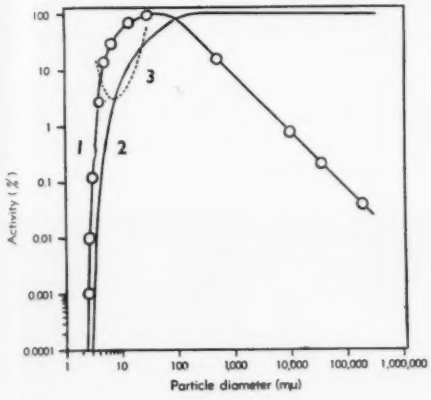


Fig. 3. Relative activity of silica as a function of particle diameter. 1, activity/gm; 2, activity/cm.^{2}; 3 (interrupted curve), activity/cm.^{2} of dried gel.

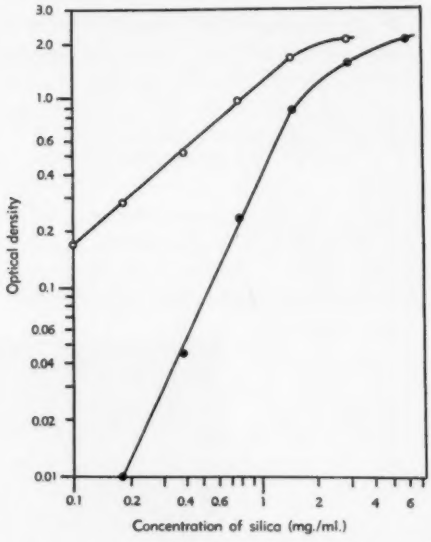


Fig. 4. Turbidity patterns of mixtures of colloidal silica (7 mμ) with citrated plasma (●) or 4 p.c. gamma globulin (○). Optical density measured at 660 mμ after 30 min. incubation.

ultimate particle size decreases from 30 to 7 mμ. Below 7 mμ, however, the activity rises again. The possible reasons for this will be discussed below.

Fading of activity.

The sudden fading of activity below a certain concentration of the smaller particle colloid (see Fig. 2) was mentioned in connection with the quantitative measurements. The simplest explanation of this phenomenon would be the dissolution of the particles in the medium. It is, however, known that this process is very slow (Iller, 1955). In a model experiment in which the haemolysis of washed red cells was used as an indicator (Harley and Margolis, 1961), 7 mμ particles were incubated for many hours, even in much greater dilutions than those used for coagulation, yet there was no evidence of any decrease in the particle size. Addition of citrate and calcium to the system had no significant effect.

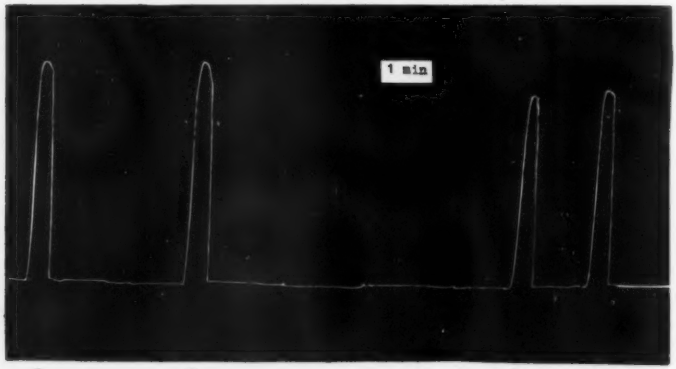
A physical counterpart of the fading of activity was found in the changes in turbidity. The plasma was incubated with serial dilutions of the 7 mμ particles and the optical density read after 30 min. In parallel, a similar series of tubes with 4 p.c. solution of human gamma globulin was used as a control. It will be seen in Fig. 4 that the optical density of the gamma globulin decreases

in proportion to the concentration of silica, whereas with plasma the fall is much steeper below 1 mg./ml. silica concentration.

It seems probable that the fading of activity is not a simple physical artefact but is due to some plasma component which can somehow "neutralise" a certain amount of colloidal silica.

Release of plasma kinin.

It has been shown elsewhere that the initial stages of the reactions leading to clotting and to the release of plasma kinin take place in common (cf. Margolis, 1959b). We may therefore expect that the effect of colloidal silica would be the same in both systems. The results in Fig. 5 confirm this view.



Latent period (sec.)	6		5				12	8
Silica particle diameter (mμ)	5,000	7	7	2	2	2	2 + 7	2 + 7
Concentration of silica in plasma (mg./ml.)	10	0.1	0.3	0.3	1.0	2.5	1.2+0.5	0.5+0.5

Fig. 5. Contractions of rat uterus in 10 ml. oxygenated Ringer's solution after addition of 0·1 ml. plasma activated for 2 min. with silica suspensions.

DISCUSSION.

Proteins adsorbed on to solid/liquid interfaces often undergo denaturation (Fraser, 1957). There is much evidence in favour of the view that the first stage in the activation of plasma by contact is the adsorption and partial denaturation of a specific globulin called the Hageman factor (Ratnoff and Colopy, 1955). More explicitly, this entails some degree of unfolding of the globulin. The exact dimensions of the molecules are not known, but they are probably larger than 10 mμ. In order to unfold, the protein must be attached to the surface in at least two points separated by an adequate distance. On purely geometrical grounds this is clearly impossible when the units of the adsorbing

surface are considerably smaller than the individual protein molecules, as in the case of small particle silica dispersions. In this connection it is interesting to note that weight for weight bentonite is a more powerful activator of plasma than is colloidal silica (unpublished observations). This mineral consists of thin platelets (1 mμ thick) of montmorillonite, in which the flat surfaces are almost pure silica. Thus, because of its crystal shape, bentonite combines a very large specific surface area with a sufficiently large unit of adsorbing surface.

At first sight, the behaviour of dry gels (Fig. 3, curve 3) may appear inconsistent because as the ultimate particle size decreases below 7 mμ the activity rises again. However, aggregates of the smallest silica spherules (e.g. 3 mμ) at the surface of the gel may act as a contiguous surface, whereas with 7 mμ particles the surface may be too irregular for the purpose. In gels made from 30 mμ sols the arrangement of the units does not matter because each particle itself acts as an efficient denaturation site. This hypothesis is represented schematically in Fig. 6. The geometrical interpretation is, of course, only a working hypothesis and rests on the assumption that there are no relevant differences other than size between the large and small silica particles.

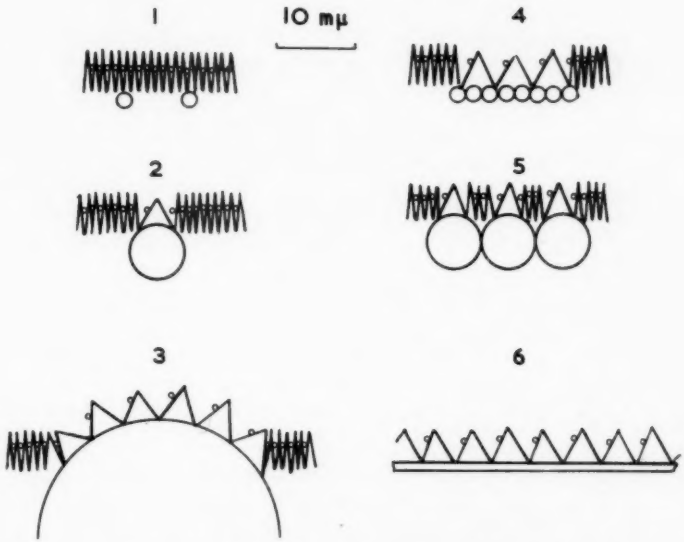


Fig. 6. Schematic representation of the hypothetical relation between the adsorbed protein (Hageman factor) and silica. The small circles between the folds represent enzymatically active sites which become exposed on unfolding of the protein molecules. (Each molecule probably has only one or two active sites. In this diagram multiple sites were drawn to represent the statistical results of activation of many molecules.) 1, 2, 3 = colloidal silica. 4, 5 = dried gel. 6 = bentonite.

The "fading" of activity has not yet been studied in detail, but the following features deserve attention. (1) It becomes pronounced only with the smaller colloidal particles (cf. Fig. 2). (2) A specific plasma component may be involved, because "fading" does not occur in model systems such as gamma globulin or red cell suspensions. The observations on turbidity changes (Fig. 4) suggest that it may be in some way connected with the problem of "colloidal stability" of plasma and not confined to coagulation alone.

The experiments with rat uterus are of little more than formal significance. Since there is not much doubt that activation of Hageman factor is the common initial step in both coagulation and the release of plasma kinin (Margolis, 1959b), it is not surprising that the surface conditions should have the same effects in both these systems.

Apart from purely academic interest, the present observations may have practical applications. (1) As was mentioned before, the coagulation methods could be applied as a very sensitive measure of particle size in the range 2-5 mμ. To do so it would first be necessary to callibrate accurately the curves in Fig. 3 with very uniform and well-specified preparations of colloidal silica. (2) If the concept illustrated in Fig. 6 is valid then the very small silica particles could be used for precipitation of certain proteins in their native state. (3) The "fading of activity" and the peculiarities in precipitation (Fig. 4) may have a bearing on the understanding of some flocculation reactions such as bentonite flocculation test (Bozicevich *et al.*, 1958).

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THE ACTION OF PHOSPHATIDASE A, SODIUM OLEATE AND GANGLIOSIDE ON THE EXOTOXINS OF *CL. WELCHII* AND ON THE NEUROTOXIN OF *SHIGELLA SHIGAE*

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SUMMARY.

The lethal effect of the exotoxins of *Clostridium perfringens* and *Cl. ovitoxicum* in mice is antagonised by phosphatidase A. In this respect, and in the fact that they are inactivated by sodium oleate and ganglioside *in vitro*, these toxins behave like those of Gram-positive bacteria previously studied. By contrast phosphatidase A, sodium oleate and ganglioside do not protect against *Shigella shigae* neurotoxin. Previous work had indicated that inactivation of toxins by both sodium oleate and ganglioside was in the nature of non-specific adsorption to protein, whilst Shiga toxin has been proved to be a protein. Comment is made on these and other points of interest.

INTRODUCTION.

Recent studies (North and Doery, 1958a, b; North and Doery, 1960a, b) have shown that the effect of the enzyme phosphatidase (lecithinase) A, which is a component of the venom of certain snakes and insects, is antagonistic to the action of staphylococcal, diphtheria and tetanus toxins in mice. It has also been demonstrated (Doery and North, 1960; North and Doery, 1960b) that the products of the action of this enzyme on lecithin, namely unsaturated fatty acid and lysolecithin, are also protective. Ganglioside prepared from ox brain inactivates all three toxins *in vitro*. Lysolecithin will release ganglioside when added to brain homogenate (Doery, 1960). This fact prompted a suggestion (North and Doery, 1961) that lysolecithin may act by releasing ganglioside *in vivo*.

The alpha toxin of *Cl. welchii A* is known to be the enzyme phosphatidase C (Macfarlane and Knight, 1941). It was therefore of interest to investigate the effect of phosphatidase A, sodium oleate and ganglioside on the lethal action of *Cl. welchii* toxins in mice.

Also it seemed of interest to study the effect of these same substances on the neurotoxin of *Shigella shigae*. Since this material may be separated easily from the cell bodies it is usually referred to as Shiga exotoxin, although there is no evidence that it is actually excreted by the living cells (Cheever, 1958).

MATERIALS AND METHODS.

Phosphatidase A.

This was separated from the dried crude venom of the black snake (*Pseudechis porphyriacus*) (Doery and Pearson, 1961). In some experiments the dried crude venom of the black snake (B.S.V.) was used.

Sodium oleate.

Oleic acid was dispersed in 0·1 N NaOH with warming (3 p.c. w/v.) and the pH adjusted to 7·3-7·5. It was then diluted with normal saline to the appropriate concentration as required.

Ganglioside.

Crude ganglioside was prepared from the grey matter of ox brain by the method described by Bogoch (1958) and purified by solvent partition in the proportions of 100 mg. in chloroform: methanol (2:1 v/v) 20 ml. and 0·1 M NaCl, 5 ml. The aqueous layer was dialysed and then freeze dried. An aqueous solution (1-10 mg./ml.) was held in a stoppered pyrex tube at 80° C. for 10-15 minutes. The aqueous solution was then diluted to the strength required with normal saline or with Palitsch borate buffer diluted 1:5 with normal saline (Macfarlane, 1950) for tests against *Cl. welchii*, type A toxin.

Cl. welchii, *type A* (*perfringens*) *toxin.*

This was a crude dried toxin, C832, supplied by Mr. Rasmussen.[1] An 8-hr. culture of S107A was Seitz filtered and sufficient ammonium sulphate to make a 60 p.c. solution (w/v) was added to the filtrate. The precipitate which collected overnight as a scum was removed and dried in a desiccator over P_2O_5. Mr. R. W. Patterson, B.Sc.,[2] partially purified the preparation by re-precipitation with ammonium sulphate as above. The precipitate was dialysed overnight to give a concentrated solution of toxin.

Cl. welchii, *type D* (*ovitoxicum*) *toxin.*

This was also a crude dried toxin (prepared in the same way as the *Cl. welchii* A toxin) supplied by Mr. W. Fallon.[3]

Shigella shigae *toxin.*

This was prepared by the method of Dubos and Geiger (1946) from a "rough" strain, type I, 9718, supplied by Mr. C. Glenn.[4]

Briefly, the method consisted of growing the organism for 36 hours under extreme aerobic conditions at pH 7·0 in a simple medium free of organic iron and containing fumaric acid, glucose, meat extract and peptone. After standing for a further three days at 37° without agitation the pH was adjusted to 4·2 and the precipitate allowed to settle. This was separated by centrifugation and the toxin extracted with phosphate buffer saline at pH 7·6. It was sterilized by filtration through sintered glass and the pH adjusted to 6·8.

In contrast to the experience of Dubos and Geiger (1946) we found no loss of activity after 5 min. at 60° C. at pH 9·0, but inactivation when held at 90° C. for 15 minutes at pH 6·4. There was no loss of activity after storage at 2° C. for some months or after freeze drying.

Haemolysin titrations of toxin of Cl. welchii *type A.*

Tests were carried out by the method of Macfarlane (1950) using Palitsch borate buffer diluted 1:5 in saline for making toxin dilutions and sheep's red cell suspensions. The mixtures

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1, 2, 3, 4 On the staff of these laboratories.

of red cells and toxin dilutions were held at 37° C. for 30 minutes. The tubes were then placed at 2° C. and the results read 15 minutes later.

Experimental animals.

White mice weighing 16-18 gm. were used for both protection and toxicity tests.

Administration of toxins and protective agents.

The methods were those used previously (North and Doery, 1961). All injections were given intravenously unless otherwise stated. In protection tests the volume of each injection was 0·5 ml.

Toxicity (LD_{50}) tests of bacterial toxins, etc.

Groups of four mice each were injected with graded doses (50 p.c. increments) within the appropriate range.

RESULTS.

Protection tests against Cl. welchii *type A (perfringens) toxin in mice.*

A protective effect could be shown regularly when this toxin and phosphatidase A were mixed immediately before injection. The results of a representative experiment are shown in Table 1. However, the impression was gained

TABLE 1.

Antagonism between the action of the phosphatidase A and the lethal effect of Cl. welchii *type A toxin in mice.*

Material injected I.V. into mice	Day of death	Survivors 7 days after challenge
Cl. welchii type A toxin alone	1, 1, 1, 1, 1, 1, 4	3/10
Toxin + phosphatidase A 0·0025 mg.	2	9/10
Toxin + phosphatidase A 0·005 mg.	1	9/10

Toxin and phosphatidase A were mixed immediately before injection.

that an additive toxic effect was more likely to occur when *Cl. perfringens* toxin and phosphatidase A were injected together than when a mixture of phosphatidase A and staphylococcal toxin was administered. In the experiment shown in Table 1, 0·005 mg. of enzyme represents less than one-fifth of an LD_{50} yet this was the maximum amount tolerated. Injected together with lethal amounts of staphylococcal toxin as much as one-quarter of an LD_{50} of phosphatidase A gave statistically highly significant protection (North and Doery, 1960a).

Cl. welchii type A toxin was inactivated by sodium oleate *in vitro* (Table 2). It will be noticed that nearly two fatal doses of toxin are neutralized by 0·6 mg. of sodium oleate in 30 minutes at 37° C.

Early attempts to show inactivation of toxin by ganglioside in which all dilutions of *Cl. perfringens* toxin and ganglioside were made in normal saline

TABLE 2.

Inactivation of Cl. welchii *type A toxin by sodium oleate.*

Material injected I.V. into mice	Day of death	Survivors 7 days after challenge
Cl. welchii type A toxin alone (0·0625 mg.)	1, 1, 1, 1, 1, 1, 1, 3, 3	1/10
Toxin (0·125 mg.)+sodium oleate (0·6 mg.)	1	9/10
Toxin (0·0625 mg.)+sodium oleate (0·6 mg.)		10/10

Cl. welchii type A toxin held with sodium oleate at 37° C. for 30 minutes before injection.

failed completely. In fact, ganglioside appeared to enhance the action of the toxin. The crude toxin of *Cl. perfringens* is (on a weight basis) very weak, the LD_{50} by the I.V. route for mice (16-18 gm.) being in the order of 0·05 mg. (see Table 2). As ganglioside interacts with proteins other than toxins (North and Doery, 1961) its effect on a concentrated and refined toxin was studied. The results were similar to those obtained using the crude toxin.

However, it was subsequently found that ganglioside (0·4 mg./ml.) in Palitsch borate buffer diluted 1:5 in isotonic saline (Macfarlane, 1950) reduced the hot-cold haemolytic titre of the toxin (0·1 mg./ml.) to one-quarter of its original value.

When mouse protection tests were carried out with toxin and ganglioside diluted in borate buffer as for the haemolysin titrations ganglioside inactivated *Cl. perfringens* toxin to some extent. The protocol of one such experiment is shown in Table 3. It will be noted that the protective effect of the ganglioside is more definite against the smaller challenge dose of toxin. The difference in mortality between animals treated and not treated with ganglioside is, in this case, not statistically significant. However, the same trend was seen in all other tests, in one of which a significant degree of protection was noted.

TABLE 3.

Inactivation of Cl. welchii *type A (perfringens) toxin* in vitro *by ganglioside.*

Material injected I.V. into mice	Day of death*	Survivors 7 days after challenge
Cl. perfringens toxin 0·04 mg.	1, 1, 1, 1, 1, 1, 1, 2, 2, 2, 2	9/20
Cl. perfringens toxin + ganglioside 0·5 mg.	3, 4	8/10
Cl. perfringens toxin + ganglioside 0·17 mg.	1, 1, 1, 1	6/10
Cl. perfringens toxin 0·045 mg.	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1	3/20
Cl. perfringens toxin + ganglioside 0·5 mg.	1, 1, 1, 1, 2, 2, 2, 3	2/10
Cl. perfringens toxin + ganglioside 0·17 mg.	1, 1, 1, 2, 7	5/10

The toxin dilutions and toxin-ganglioside dilutions—all in Palitsch buffer saline—were held at 37° C. for 1¾ hours before injection into mice.

Protection tests against Cl. welchii *type D (ovitoxicum) toxin in mice.*

The results of two representative experiments (Table 4) show that there is some antagonism between the action of phosphatidase A and *Cl. ovitoxicum* toxin.

In test No. 1 (Table 4) the balance between the dose of toxin and phosphatidase A was such that the only evidence of protection is in some prolonga-

TABLE 4.

Antagonism between the action of phosphatidase A and the lethal effect of Cl. welchii *type D (ovitoxicum) toxin in mice.*

Test No.	Material injected I.V. into mice	Time of death*	Survivors 7 days after challenge
1	*Cl. ovitoxicum* toxin alone (0·0037 mg.)	1, 1, 1, 1, 1, 1, 1, 1, 1, 2	0/10
	Toxin + phosphatidase A (0·002 mg.)	1, 1, 1, 1, 3, 3, 4, 5, 7,	1/10
	Toxin + phosphatidase A (0·004 mg.)	1, 1, 1, 1, 1, 1, 1, 2, 4, 6	0/10
2	*Cl. ovitoxicum* toxin alone (0·0035 mg.)	1, 1, 1, 1, 1, 1, 1	3/10
	Toxin + B.S.V. (·001 mg.)	1, 1, 3	7/10
	Toxin + B.S.V. (·002 mg.)	1, 1, 1, 1, 4	5/10
	Toxin + B.S.V. (·004 mg.)	h, 1, 1, 1, 1, 1, 1, 5, 6, 6	0/10

In test No. 2 crude B.S.V. was used as the source of phosphatidase A.

* The letter "h" indicates death within 4 hr. and the numerals the day of death after challenge.

tion of life of the treated mice. However, in test No. 2, in which the mice were challenged with a slightly smaller dose of toxin, a well-graded degree of protection is seen. It may be noted that a dose of 0·004 mg. of crude black snake venom enhanced the lethal effect of the toxin, whilst 0·001 mg. afforded some definite protection.

TABLE 5.

Inactivation of Cl. welchii *type D (ovitoxicum) toxin* in vitro *by sodium oleate and by ganglioside.*

Treatment of mice		Time of death*	Survivors 7 days after challenge
Material injected I.V.	Treatment prior to injection		
Cl. ovitoxicum toxin alone ·004 mg.	Fresh	h, h, h, h, h, h, h, h, 1, 1	0/10
Cl. ovitoxicum toxin alone ·004 mg.	Held overnight on bench	h, h, h, h, 1, 1, 1, 1, 1, 3	0/10
Cl. ovitoxicum toxin alone ·004 mg.	37° C. 4 hours	1, 1, 1, 1, 1, 3, 3, 4, 7, 7	0/10
Cl. ovitoxicum toxin + sodium oleate (0·6 mg.)	37° C. 30 min.		10/10
Cl. ovitoxicum toxin + ganglioside (0·25 mg.)	37° C. 4 hours		10/10

* The letter "h" indicates death within 4 hours and the numerals the day of death after challenge.

Unlike its adverse effect on *Cl. perfringens* toxin when saline was the diluent, ganglioside in saline inactivated *Cl. ovitoxicum* toxin. The protocol of one experiment is given in Table 5, together with that of another test with sodium oleate as the protective agent set up at the same time. It will be seen that the *Cl. ovitoxicum* toxin is moderately stable.

Protection tests against Shigella shigae *neurotoxin in mice.*

Phosphatidase A appeared to enhance rather than antagonise the lethal effect of the toxin. Sodium oleate and ganglioside had no effect, either favourable or unfavourable, on the neurotoxin under the conditions in which they were used. Protocols of representative experiments are shown in Table 6.

TABLE 6.

Ineffectiveness of phosphatidase A, sodium oleate and ganglioside against action of Shigella shigae *toxin in mice.*

Treatment of *Shigella shigae* toxin before injection I.V. into mice	Day of death	Survivors 7 days after challenge
Toxin (1:40) 0·5 ml. alone	3, 3, 3, 3, 4, 4, 5, 5, 5	1/10
Toxin + phosphatidase A (0·00075 mg.)	2, 2, 2, 2, 2, 2, 2, 2, 3, 3	0/10
Toxin + phosphatidase A (0·0015 mg.)	1, 2, 2, 2, 2, 2, 2, 2, 3, 3	0/10
Toxin (1:10) 0·5 ml. alone	1, 2, 3, 3, 3, 3, 3, 4, 5, 5	0/10
Toxin (1:10) + sodium oleate 0·6 mg. held at 37° C. 30 mins.	2, 2, 3, 3, 3, 3, 3, 4, 4, 4	0/10
Toxin (1:20) + sodium oleate 0·6 mg. held at 37° C. 30 mins.	2, 3, 3, 3, 3, 3, 3, 3, 4, 5	0/10
Toxin (1:40) + sodium oleate 0·6 mg. held at 37° C. 30 mins.	3, 3, 4, 4, 5, 5, 6	3/10
Toxin (1:40) 0·5 ml. alone	2, 3, 3, 3, 3, 3, 4, 5, 5, 7	0/10
Toxin (1:40) + ganglioside 1 mg. held at 37° C. 30 mins.	2, 2, 2, 2, 2, 3, 4, 4, 5, 5	0/10
Toxin (1:40) + ganglioside 1 mg. held at 37° C. 3½ hours	2, 3, 3, 3, 3, 3, 3, 4, 7	1/10

The mixtures of toxin + enzyme were injected immediately after preparation.

DISCUSSION.

The results reported in this paper show that the lethal effect of the exotoxins of *Cl. perfringens* and *Cl. ovitoxicum* is antagonised by the action of phosphatidase A in mice and that sodium oleate completely inactivates these toxins *in vitro*. In both respects these clostridial toxins, therefore, resemble the exotoxins of Gram-positive bacteria studied previously, namely, *Staphylococcus, C. diphtheriae* and *Cl. tetani* (North and Doery, 1960a; Doery and North, 1960).

The *Cl. welchii* toxins have also been shown to follow still further the pattern already established for the toxins of these other Gram-positive organisms (North and Doery, 1960b and 1961) in that they are inactivated by ganglioside although under the experimental conditions used *Cl. welchii* type A toxin was relatively less susceptible to ganglioside than the others.

Cl. welchii type A toxin contains neuraminidase (McCrae, 1947) and this enzyme has also been found in the toxin of *Cl. welchii* type D used in these

experiments. However, it has been shown that the previous interaction of ganglioside and neuraminidase with the release of 17 p.c. of the weight of the former as free sialic acid does not affect the inactivation of staphylococcal toxin by the ganglioside (to be published). Thus, it seems unlikely that the difference in susceptibility of these two welchii toxins to the action of ganglioside is related to their content of neuraminidase.

Since sodium oleate interacts non-specifically with proteins, including serum protein (Dubos and Davis, 1946; Doery and North, 1960), and crude venoms of some snakes (North and Doery, 1960, unpublished) and gangliosides combine with serum proteins under the experimental conditions used (North and Doery, 1961), it was considered that the interaction of the bacterial toxins with ganglioside and sodium oleate was also non-specific.

It has already been suggested (Doery and North, 1960; North and Doery, 1961) that phosphatidase A protects against the action of bacterial toxin *in vivo* through the release of long chain fatty acids and lysophosphatides which, in turn, may release ganglioside.

Whatever the mechanisms involved, the complete failure of phosphatidase A, sodium oleate and ganglioside to protect against the neurotoxin of *Shigella shigae* is of interest. This neurotoxin has been isolated as a highly purified protein but the mechanism by which it is produced is unknown (van Heyningen and Gladstone, 1953). It is usually referred to as Shiga exotoxin but there is no evidence that it is actually excreted by the living cell (Cheever, 1958). The fact that it is not inactivated by sodium oleate suggests that this protein differs in some essential way from the proteins of the exotoxins of the Gram-positive bacteria and crude venoms of Australian snakes we have examined.

Another point of interest in our findings is the fact that the action of phosphatidase A can be shown to antagonise the lethal effect of *Cl. perfringens* exotoxin. The alpha toxin of *Cl. perfringens* is an enzyme, lecithinase C, which acts on the same substrate as lecithinase A. Whilst the latter splits off a fatty acid from lecithin leaving lysolecithin, lecithinase C splits off phosphorylcholine leaving a diglyceride. Lecithinases are also produced by other clostridia (Sterne and van Heyningen, 1958) and phosphatides are present in all animal tissues. The results of the competition for the same substrate between lecithinase A and other lecithinases excreted by the clostridia in infected wounds may well be an important factor as to whether gas gangrene does or does not occur.

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RESISTANCE VALUES IN A SYNCYTIUM

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SUMMARY.

Cable theory has been extended to include the case where the cable divides into two after a distance l, the two branches again dividing and so on indefinitely. This is referred to as the open syncytium. The electrical behaviour is dependent on the ratio l/λ : for $l/\lambda >> 1$, $R \propto r_m^{0.5}$ where λ is the attenuation length, R is the input resistance and r_m the membrane resistance; for $l/\lambda << 1$ it is found that R is constant. For intermediate values of l/λ the dependence of R on r_m lies between these two limits.

The case has also been considered where some of the branches loop back into parental branches. This has been called the closed syncytium. An extreme example of the closed syncytium is where the loops form a close-packed hexagonal array. In this case, for $l/\lambda >> 1$, $R \propto r_m^{0.5}$ as for the open syncytium. However, for $l/\lambda << 1$, there is a significant difference between the two structures and now R is not constant but $R \propto r_m^{0.25}$ approx.

INTRODUCTION.

The fibres of cardiac muscle have been assumed to form a syncytium. However, recent electron microscopy (Muir, 1957a, b) has shown that histologically cardiac muscle is not a syncytium as each fibre is separated from the next in the region of the intercalated disc. Weidmann (1952), in measuring the space constant of a long column of kid Purkinje fibre cells (which have intercalated discs), found that they behaved as a single core cable when current was injected at a point. He found no discontinuity in the potential distribution along the fibre as would occur if the intercalated discs formed high resistance barriers. Draper and Mya-Tu (1959), studying conduction of the action potential in atria, ventricles and Purkinje system of the hearts of different species, state that their results did not support the view that the intercalated disc is a barrier to longitudinal current flow within the fibre. It is important therefore to consider the case of the fibres behaving electrically as a syncytium.

The theory of the electric telegraph originally formulated by Lord Kelvin is frequently applied to electrically active tissue (Hodgkin and Rushton, 1946). In doing so, it is assumed that the tissue in question behaves as a single cable-like structure. The present paper is concerned with the extent to which the same theory can be applied to cardiac muscle. Only steady values of current and voltage will be considered in this paper and the question of the transient

response will be dealt with separately. The main interest is in the connection between the input resistance at a point, R, and the leakage resistance per unit length, r_m, of the cables, this latter corresponding to the membrane resistance of the syncytial cells. It is well known that for a single cable

$$R \propto r_m^{0.5} \quad \ldots\ldots \quad (1)$$

but it is found that in syncytium R is less dependent on r_m than is implied by equation (1).

In the following discussion the model syncytium will be taken as being made up of identical lengths of leaky cable of length l. At each end of the unit of length l, the cable is joined to two identical units as in Fig. 1. Two cases will be considered: firstly, where the syncytial cells branch indefinitely without re-entry as in Fig. 1a, and secondly, where they connect back on themselves making closed loops as in Fig. 1b. These two alternative arrangements will be referred to as the "open" and "closed" syncytia respectively. Fig. 1b, in which the closed loops form a hexagonal array, is an extreme example of a closed syncytium.

The actual structure, if it is electrically a syncytium at all, should lie between these limits. Resistance values have therefore been calculated for these two extremes. It is found that over the range of practical importance the difference between these two models is not very great, and hence the true answer is bracketed within quite small limits. It is everywhere assumed that the syncytium is large compared with the attenuation length and that the resistance of the extracellular fluid surrounding the cells is negligible.

THEORY.

The open syncytium.

Given that the dimensions of the syncytium are large compared with the attenuation length λ it follows that the resistances at all branch points, such as A in Fig. 1a, will be equal. Let this resistance be R_0.

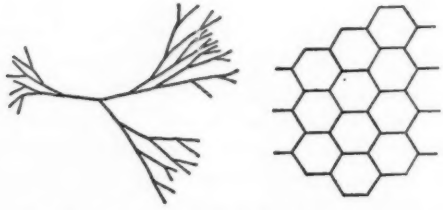

Fig. 1. a, Open syncytium; b, closed syncytium.

The input resistance, r_i, of a length l of cable terminated in an arbitrary resistance r is

$$f(l, r) = z \frac{r \cos h\, l/\lambda + z \sin h\, l/\lambda}{z \cos h\, l/\lambda + r \sin h\, l/\lambda} \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (2)$$

where $\lambda = \sqrt{r_m/r_i}$ ... ... (2·2)

$$z = \sqrt{r_m\, r_i} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (2{\cdot}3)$$

r_i = series resistance/cm.
r_m = leakage resistance/cm.

Consider one of the bits of cable in Fig. 1a. Its input resistance must be $3R_o$ when it is terminated in the resistance $1{\cdot}5\ R_o$. Hence from (2).

$$3\,R_o = z \frac{1{\cdot}5\,R_o \cos h\, l/\lambda + z \sin h\, l/\lambda}{z \cos h\, l/\lambda + 1{\cdot}5\,R_o \sin h\, l/\lambda} \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (3)$$

the solution of which is

$$R_o = \frac{-z \cos h\, l/\lambda + z\sqrt{\cos h^2 (l/\lambda + 8 \sin h^2)(l/\lambda)}}{6 \sin h\, l/\lambda} \quad \ldots\ldots \quad (4)$$

If we consider an arbitrary point at a distance x from a branch point, the resistance measured at this point would be

$$R_o\,(x) = \frac{f(x, 1{\cdot}5\,R_o) \times f(l-x, 1{\cdot}5\,R_o)}{f(x, 1{\cdot}5\,R_o) + f(l-x, 1{\cdot}5\,R_o)} \quad \ldots\ldots \quad \ldots\ldots \quad (5)$$

i.e. the resistances $f(x, 1{\cdot}5\,R_o)$ and $f(l-x, 1{\cdot}5\,R_o)$ in parallel. $f(x, r)$ is defined in equation (2). Equation (5) is the general solution to the problem, while equation (4) gives the resistance at a branch point, both for an open syncytium.

Numerical values, open syncytium.

Of interest is the dependence of the input resistance on the leakage resistance per unit length r_m. This is determined by the value of l/λ. If $l/\lambda << 1$ the dependence is different from that obtained when $l/\lambda >> 1$ and these cases will first be considered.

(a) $l/\lambda >> 1$.

From (4) the resistance at a branch point is

$$R_o(o) = 2z/3 = 2/3\ \sqrt{r_m\, r_i} \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (7)$$

while half-way between two branch points we find

$$R_o(l/2) = z/2 = 1/2\ \sqrt{r_m\, r_i} \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (8)$$

Thus when the attentuation length is short compared with the length of each cable, the solution reduces to the well-known case of a single long, leaky cable, and relation between input resistance and r_m is as in equation (1).

(b) $l/\lambda << 1$.

In this case, (4) becomes

$$R_o(o) = 1/3\, zl/\lambda$$

$$\text{or } R_o(o) = 1/3\, r_i l \quad \ldots\ldots\ldots\

The closed syncytium.

The first step in the solution for the closed syncytium is to replace each length of cable by its equivalent T-network. Thus, the leaky cable of length l is equivalent to the network in Fig. 3.

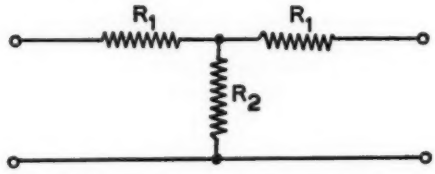

Fig. 3. Equivalent lumped network.

provided that

$$R_1 = z(\cos h\, l/\lambda - 1)/\sin h\, l/\lambda \quad \ldots\ldots \quad (11)$$

$$R_2 = z/\sin h\, l/\lambda \quad \ldots\ldots \quad (12)$$

Thus the closed syncytium (Fig. 1b) becomes a honeycomb of simple resistances. Each series resistance has the value $2R_1$ and is shunted to ground at its midpoint by the resistance R_2. A considerable reduction in the complexity of this problem can be achieved by making use of the symmetry properties of the structure.

Referring to Fig. 4, we start at some arbitrary branch point in the network and call it point 0. The distal ends of the three resistances meeting here we call 1. If current is injected at point 0, the points "1" will all be at the same potential and may therefore be connected together without causing any disturbance. Similarly the six points "2" will all be connected together and so on. Proceeding in this way, we can reduce the syncytium to the equivalent network shown in Fig. 5.

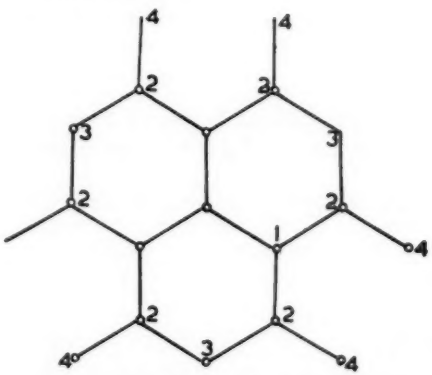

Fig. 4. Closed syncytium.

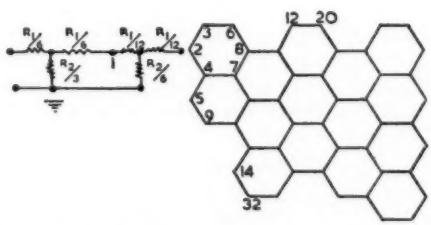

Fig. 5. Circuit equivalent to the closed syncytium. In the honeycomb structure, all the series resistances have the value R 1/6 and are grounded at their midpoints by a resistance of R 2/6 except for the external branches, 3-6, 12-20, 5-4, 14-32, etc., where the series resistances are R 1/3 and the shunt resistances R 2/3.

The other device found useful is to replace the more remote elements of the network by a continuum (Fig. 6).

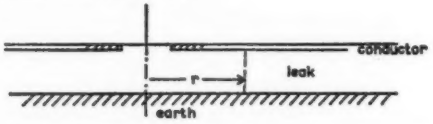

Fig. 6. Continuum used as approximation to outer layers of closed syncytium.

Let V = potential at radius r.
I = total radial current at r.
ρ = resistance of 1 sq. cm. of upper conductor.
σ = conductance cm.$^{-2}$ between conductor and earth.

The equations are:

$$dI/dr = -2\pi V\sigma r \quad \ldots\ldots \quad (13)$$

$$dV/dr = -\rho I/2\pi r \quad \ldots\ldots \quad (14)$$

Hence,

$$d^2V/dr^2 + 1/r\, dV/dr - \sigma\rho V = 0 \quad \ldots\ldots \quad (15)$$

and

$$d^2I/dr^2 - 1/r\, dI/dr - \sigma\rho I = 0 \quad \ldots\ldots \quad (16)$$

These are forms of Bessel's equation and the solutions which behave in the right way at the boundaries are:

$$V = iV_oH_o(i\sqrt{\sigma\rho}r) \quad \ldots\ldots \quad (17)$$

$$I = -2\pi r V_o\sqrt{\sigma}/\rho H_1(i\sqrt{\sigma\rho}r)$$

where $H_o(ix)$ and $H_1(ix)$ are Hankel functions of order zero and one respectively. V_o is a constant to be adjusted depending on the conditions at the inner boundary.

The resistance from a ring of radius r extending outwards to infinity will be:

$$R_c = V/I = \frac{i\sqrt{\rho/\sigma}}{2\pi r}\,\frac{H_o(i\sqrt{\sigma\rho}r)}{H_1(i\sqrt{\sigma\rho}r)} \quad \ldots\ldots \quad (18)$$

The procedure is now to fill in the inner area with the actual network (Fig. 5) and to replace the outer layers with the resistance of the equivalent continuum (18).

It only remains to express the continuum parameters ρ and σ in terms of R_1 and R_2. These are found to be:

$$\sigma = \frac{2}{R_2\sqrt{3}\,l^2} \quad \ldots\ldots \quad (19)$$

$$\rho = \frac{18\,R_1}{(3+2\sqrt{3})} \quad \ldots\ldots \quad (20)$$

where l is the length of one side of the hexagonal structure. In what follows we have set $l = 1$. This involves no loss of generality.

Hence $\sigma = 1{\cdot}152/R_2$, $\rho = 1{\cdot}39\,R_1$ and

$$R_c = \frac{iR_2\,1{\cdot}1\sqrt{K}}{2\pi r}\,\frac{H_0(i\,1{\cdot}267\sqrt{K}r)}{H_1(i\,1{\cdot}267\sqrt{K}r)} \quad \ldots\ldots \quad \ldots\ldots \quad (21)$$

where $K = \dfrac{2R_1}{R_2}$.

Numerical values, closed syncytium.

As a first approximation the inner network is terminated at meshpoint 2 in Fig. 4 and the remainder of the network is replaced by the resistance R_c given by equation (21) with $r = \sqrt{3}$.

Thus we require the input resistance of the following network:

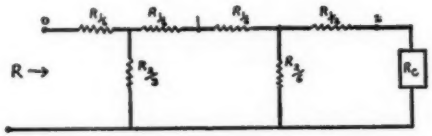

Fig. 7. Equivalent network, approximation 1.

where R_1 and R_2 are given by (11) and (12) and R_c by equation 2. We can then proceed to a higher approximation by terminating the inner network at meshpoint 3, where the radius $r = 3$. The various approximations were found to converge rapidly and after the third approximation the input resistance values did not differ significantly, and the series of approximations was terminated at the third.

The numerical values obtained in this way for the closed syncytium are shown in the interrupted lines in Fig. 2 for the same values of length and core diameter as used for the open syncytium.

DISCUSSION.

Fig. 2 summarises in graphical form the results of this work. All the curves in Fig. 2 have this much in common: for sufficiently small values of r_m, $R \propto r_m^{0.5}$. As r_m is increased, a point is reached where R increases less slowly with r_m and we may write approximately

$$R \propto r_m^{\alpha} \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (22)$$

where $0 < \alpha < 0{\cdot}5$. Obviously α varies slowly as r_m is increased and over the range values investigated settles down to a steady value, α which is different for the two types of structure investigated.

$$\left.\begin{array}{ll} \text{Open syncytium,} & \alpha = 0 \\ \text{Closed syncytium,} & \alpha = 0{\cdot}25 \end{array}\right\} \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (23)$$

The value of r_m at which the departure from equation 1 becomes significant

depends on the diameter and length of the elementary cables and on the specific resistance of the core.

If the dog Purkinje fibres have the dimensions given by Weidmann (1952), then for the usual value of the resting membrane resistance of 3000 Ω cm.$^{-2}$ one is situated nicely in the middle of that portion of curve (c), Fig. 2, where relation (1) holds for large positive and negative changes in membrane resistance. Also, for such tissues the question as to whether one is dealing with the open or the closed structure becomes irrelevant.

However, for a ventricular structure of the dimensions given by Johnson (private communication) the situation is quite different (curves (a) and (b), Fig. 2). Here the relation between R and r_m is highly dependent on the dimensions and on the overall arrangement. Taking the smaller cable length first (curve (b)) and again assuming $R_m \sim 3000\Omega$ cm.$^{-2}$, R is constant for the open structure and $R \propto r_m^{0.25}$ for the closed. For the larger ventricular structure (curve a) at $r_m = 3000\ \Omega$, intermediate values of a apply. For the open structure at this value of r_m, $R \propto r_m^{0.1}$, while for the closed structure $R \propto r_m^{0.25}$.

It is clear, therefore, that in order to be able to convert the experimentally determined values of R in ventricular muscle into corresponding values of r_m it is necessary to know not only the dimensions and electrical resistance of the elementary fibres of the tissue, but also whether the overall structure is electrically a branching network, and if so whether it is of the open or closed variety. In the case of ventricular fibres, the histological appearance suggests that the hexagonal closed packed array is the closest approximation.

Acknowledgments. I am grateful to John Tille for drawing my attention to the importance of the closed structure and to Dr. C. Wallace for suggesting the approximation of the continuum used in evaluating its resistance. The problem was proposed by Dr. E. A. Johnson, to whom I am grateful for many helpful discussions.

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SOLUTIONS OF THE HODGKIN-HUXLEY EQUATIONS FOR SQUID AXON TREATED WITH TETRAETHYLAMMONIUM AND IN POTASSIUM-RICH MEDIA

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SUMMARY.

Solutions to the Hodgkin-Huxley (H-H) equations have been obtained using the fast digital computer SILLIAC. In these theoretical calculations the parameters were adjusted to correspond to the experimental conditions prevailing in a series of experiments reported by Tasaki and his collaborators, namely, (a) normal axon in potassium-enriched sea water, or (b) tetraethylammonium (TEA) treated axon in normal sea water. Under conditions (a) the theoretical solutions demonstrated a response to hyperpolarising currents, the threshold of which was dependent on the value of the outside potassium concentration. The resulting theoretical waveforms and resistance variations were similar to those observed experimentally. The effect of TEA was simulated by reducing by a factor, k, the rate of change with time of those terms in the Hodgkin-Huxley equations governing the potassium permeability. It was found that for $k < 22{\cdot}5$, the action potentials were prolonged but were not modified significantly in shape. For $k > 22{\cdot}5$, however, a plateau reminiscent of some cardiac waveforms appeared after the initial spike. The effect of hyper- and depolarising currents, of abolition pulses, and of the response to successive stimuli, was investigated. The theoretical results were found to correspond closely with the experimental observations. It is concluded that the prolonged action potential under TEA and some features of the hyperpolarising response in potassium-rich sea water can be satisfactorily accounted for within the existing framework of the Hodgkin-Huxley equations.

INTRODUCTION.

In 1952 Hodgkin and Huxley presented a mathematical formulation of the membrane current of squid axon during voltage clamp which gave the total membrane current as a function of time and voltage. By solving these equations for both propagated and non-propagated disturbances they were able to predict the quantitative behaviour of nerve axon under a variety of conditions in remarkable agreement with the results observed experimentally.

The solutions have since been extended by the use of automatic computers by Cole, Antosiewicz and Rabinowitz (1955, 1958), Cole (1958), Huxley (1959) and Fitzhugh and Antosiewicz (1959). Frankenhaeuser and Hodgkin (1957) examined the effects of Ca^{++} ions on the squid axon and summarised their

results by saying that a fivefold increase in Ca^{++} concentration caused the axon to behave as though its resting potential were 10-15 mV. lower than its actual value. Making the appropriate changes in the Hodgkin-Huxley equations and solving them, Huxley (1959) showed that these corresponding solutions showed behaviour identical to that which had been experimentally observed.

However, squid axon under a variety of abnormal environmental circumstances behaves in a manner which has been claimed to be unexplainable in terms of the Hodgkin-Huxley model. Thus, when tetraethylammonium chloride (TEA) is injected into squid axon the action potential is remarkably prolonged and the normal voltage-dependent increase in potassium permeability is either abolished or markedly delayed. In this state the action potential can be shut off or further prolonged by passing hyperpolarising or depolarising current through the membrane (Tasaki and Hagiwara, 1957).

In potassium-rich solution the squid axon loses its normal non-linear responsiveness to a depolarising current pulse. However, with hyperpolarising currents above a certain threshold intensity the axon develops large hyperpolarising responses (Segal, 1958; Moore, 1959; Tasaki, 1959). These and other related phenomena have been interpreted by Tasaki and co-workers (Tasaki and Hagiwara, 1957; Tasaki and Spyropoulos, 1958) in terms of a bi-stable state hypothesis in which the membrane is supposed to exist in one or other of two stable states.

We thought that it would be interesting to examine whether the Hodgkin-Huxley equations predicted such behaviour. We have solved the Hodgkin-Huxley equations after making modifications appropriate to the experimental conditions. We have found that these equations do predict behaviour very similar to that found with TEA and potassium-rich solutions.

Theoretical Basis.

Using the voltage clamp technique, Tasaki and Hagiwara (1957) demonstrated that in the squid axon treated with TEA the sodium-carrying mechanism is retained but that the subsequent rise in potassium conductivity is markedly retarded. For example, in normal axon under voltage clamp and with steady depolarisations in excess of 60 mV. the membrane current after a few milliseconds consists of a steady outward potassium current of more than 1 mA. $cm.^{-2}$ Inspection of the records of Tasaki and Hagiwara (1957) shows that in the TEA treated axon depolarised by 78 mV. the potassium permeability is not changed appreciably from its resting value of approximately 1×10^{-3} mhos. $cm.^{-2}$ Initially the outward potassium current is only 150 μA. $cm.^{-2}$ and is not steady but rises at a rate of approximately 1 μA. $cm.^{-2}$ $msec.^{-1}$.

We have therefore modified the Hodgkin-Huxley equations (Hodgkin and Huxley, 1952) in such a manner as to comply with these experimental conditions. For the membrane action potential the equations to be solved may be written in the well-known form (Hodgkin and Huxley, 1952).

$$I = C_M (dV/dt) + \bar{g}_K n^4 (V - V_K) + \bar{g}_{Na} m^3 h (V - V_{Na}) + \bar{g}_l (V - V_l) \quad (1)$$

I is the membrane current per unit area and is equal to zero, C_M the membrane capacity per unit area, $\bar{g}_K$, $\bar{g}_{Na}$, $\bar{g}_l$ are constants occurring in the expressions for the potassium, sodium and leakage currents respectively.

The remaining quantities satisfy the equations:

$$dn/dt = \alpha_n (1-n) - \beta_n n \quad (2)$$

$$dm/dt = \alpha_m (1-m) - \beta_m m \quad (3)$$

$$dh/dt = \alpha_h (1-h) - \beta_h h \quad (4)$$

In the normal axon we also have:

$$\alpha_n = 0{\cdot}01 (V+10) / \left(\exp \frac{V+10}{10} - 1 \right) \quad (5)$$

$$\beta_n = 0{\cdot}125 \exp (V/80) \quad (6)$$

$$\alpha_m = 0{\cdot}1 (V+25) / (\exp \frac{V+25}{10} - 1) \quad (7)$$

$$\beta_m = 4 \exp (V/18) \quad (8)$$

$$\alpha_h = 0{\cdot}07 \exp (V/20) \quad (9)$$

$$\beta_h = 1/(\exp \frac{V+30}{10} + 1) \quad (10)$$

V, V_K, V_{Na}, V_l are in millivolts; t is in milliseconds.

V = O corresponds to the resting value of the membrane potential, depolarisations and hyperpolarisations are presented as changes in potential in a negative and positive direction respectively.

The following numerical data were also taken from Hodgkin and Huxley (1952):

C_m (μF cm.$^{-2}$)	1·0
V_{Na} (mV.)	−115
V_K (mV.)	+ 12
V_l (mV.)	− 10·613
$\bar{g}_{Na}$ (m. mho. cm.$^{-2}$)	120
$\bar{g}_K$ (m. mho. cm.$^{-2}$)	36
$\bar{g}_l$ (m. mho. cm.$^{-2}$)	0·3
Temperature: 6·3° C.	
$[K]_o$ in normal sea water	13 mM.

We set out to solve the above equations (1) to (10) for the following assumptions:

(a) In order to simulate the effect of TEA we solved the equations using α'_n and β'_n where $\alpha'_n = \alpha_n/k$ and $\beta'_n = \beta_n/k$ and α_n and β_n are given by equations (5) and (6), thus slowing the potassium mechanism down by a factor k.

(b) $\alpha'_n = \beta'_n = 0$ which corresponds to n being constant and the potassium conductivity remaining indefinitely at its normal resting value G_K (i.e. for $V = 0$).

(c) In order to simulate the effects of potassium-rich media we solved the equations when the value of V_K in eqn. (1) was altered in accordance with the change in the extracellular potassium concentration $[K]_o$. If $[K_1]_o$ is the normal value of $[K]_o$ and $[K_2]_o$ the value in the enriched medium we changed the value of V_K by the amount ΔV_K where $\Delta V_K = 58 \log_{10} [K_1]_o/[K_2]_o$ mV.

METHODS.

The calculations were carried out with the aid of the fast digital computer SILLIAC in the Adolph Basser Computing Laboratory of the Physics Department in the University of Sydney. The Runge-Kutta method was used in order to solve the equations (1) to (4) with the conditions (5) to (10) and with the assumptions (a), (b) or (c) above.

While coding the problem for machine computation it was noted that equations (5) and (7) were indeterminate at $V = -10$ and -25 mV. respectively. It was realised that this would lead to difficulties in computation which were avoided in the following way. When $9{\cdot}9 < -V < 10{\cdot}1$ mV., the value of α_n for $V = -10$ mV., calculated independently, was substituted. This value is $\alpha_n = 0{\cdot}1$ for $V = -10$ mV. A similar procedure was adopted for α_m when V was within $0{\cdot}1$ mV. of $V = -25$ mV. The errors involved in not carrying out this manoeuvre have recently been reported by Fitzhugh and Antosiewicz (1959).

RESULTS.

TEA-treated Axon.

Effect of varying the retardation (k) of the potassium conductance mechanism.

We consider first the theoretical action potentials which result when the rate constants involved in the potassium carrying mechanism are slowed down by a factor k.

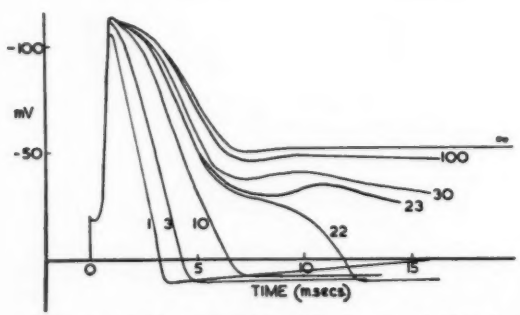

Fig. 1. Action potentials following an initial depolarising stimulus of 20 mV. at $t = 0$ for various values of the retardation factor $k = 1, 3, 10, 22, 23, 30, 100$ and ∞.

The action potentials following an initial depolarising stimulus of 20 mV. are shown in Fig. 1. The curve for $k = 1$ should correspond to the calculated curve of Hodgkin and Huxley (1952) and does in fact do so. For values of k between 1 and 22·5 the normal waveform is essentially retained. The repolarisation phase, as would be expected, becomes somewhat prolonged as the value of k is increased.

For values of k greater than 22·5 a sudden transition occurs in the waveform and the potential does not return to its resting value but remains at a value between -30 and -50 mV. depending on the value of k.

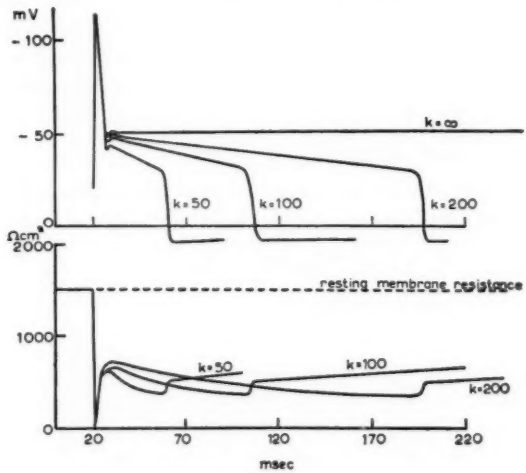

Fig. 2. Effect of slowing down the rate constants involved in the potassium-carrying mechanism by a factor k.
Upper figure: Action potential waveforms for retardation factors (k) of 50, 100, 200 and ∞.
Lower figure: Time course and magnitude of membrane resistivity during the action potentials for $k = 50$, 100 and 200 shown in the upper figure.

For $k > 22·5$ the solutions were extended over a longer time. The results for $k = 50$, 100 and 200 are shown in Fig. 2. These potentials exhibit a pronounced spike of 114 mV. in amplitude followed by a prolonged plateau which is terminated by a rapid phase of repolarisation followed by an after-hyperpolarisation or undershoot. The duration and slope of this plateau depend on the value of k. The magnitude of the undershoot is initially 8·5 mV. and decreases slowly with time. Calculated values of membrane undershoot and the corresponding values of m, h and n as a function of time are given in Table 1.

The decay of the undershoot follows closely an exponential time course, with a time constant of approximately 400 msec.

TABLE 1.

Values of membrane undershoot (V) and the corresponding values of n, m and h at varying times for k=100.

Time after stimulation (msec.)	V (mV.)	n	m	h
100	+8·33	·457	·019	·704
200	+7·03	·417	·0224	·808
300	+5·67	·387	·0266	·775
400	+4·36	·364	·0313	·739
500	+3·12	·351	·036	·705

Also shown in Figs. 1 and 2 are the results for $k = \infty$. Under this condition the action potential exhibits the same spike of 114 mV. as for other large k values, but there is no repolarisation. The membrane remains depolarised indefinitely at a potential of -52 mV.

Resistance values (normal sea water).

The instantaneous theoretical membrane conductivity, g_m, follows from equation (1) and is given by

$$g_m = \bar{g}_K n^4 + \bar{g}_{Na} m^3 h + g_l = \lim_{\Delta V, t \to 0} \left(\frac{\Delta I}{\Delta V} \right) \quad \ldots\ldots (11)$$

whereas R_m the membrane resistivity, is simply:

$$R_m = 1/g_m \quad \ldots\ldots \ldots\ldots \ldots\ldots \ldots\ldots (12)$$

In the lower part of Fig. 2 are shown the curves of membrane resistivity against time, calculated in this way, for three prolonged action potentials when $k = 50$, 100 and 200. They are characterised by a resting level of 1480 ohm. cm.^{2} and a sudden drop during the spike to 30 ohm. cm.^{2} Thereafter the resistance falls slowly throughout the plateau. During the fast phase of repolarisation there is a sudden increase in resistance attributable to the decrease in sodium conductance. Following this the resistance increases further, throughout the period of undershoot, returning slowly to the original value for the resting membrane.

Applied membrane currents.

In this section and all subsequent ones in which the potassium mechanism is retarded the value $k = 100$ has been arbitrarily chosen.

In the absence of externally applied membrane currents the right-hand side of equation (1) is set equal to zero. The effect of applied membrane currents can be very easily computed by equating the r.h.s. of equation (1) to the required current value.

The modifications to the prolonged action potentials resulting from hyperpolarising and depolarising currents are shown in Fig. 3. In these computations the applied current was switched on at the same time as the stimulus was applied.

It was found that in the plateau region of the action potential, the voltage displacement is approximately linearly dependent on the polarising current over the range investigated of ± 40 μA. cm.$^{-2}$ The effect of depolarising currents is to prolong the action potential while that of hyperpolarising currents is the reverse.

By dividing the voltage displacements by the applied currents it is possible to express the ratios as a membrane resistivity which, however, is not the same as that given by equations (11) and (12). In the plateau region this membrane resistivity is found to be 175 ohm. cm.^{2} and following repolarisation around 400 ohm. cm.^{2} These values are significantly lower than those calculated from equations (11) and (12). The difference arises from the fact that equations (11) and (12) refer to infinitely fast voltage displacements so that the parameters m, n, and h do not change during the measurement. These conditions would also apply if the membrane resistivity were measured by means of a high frequency bridge. In this case the values of m, n and h would not have *time* to change if the bridge frequency were sufficiently high. Using the method of measuring the response to prolonged polarising currents, modifications in m, n, h in response to the voltage change produced by the measuring current would have time to take effect and the membrane resistivity measured in this way is usually lower than the value obtained with high frequency bridge measurements.

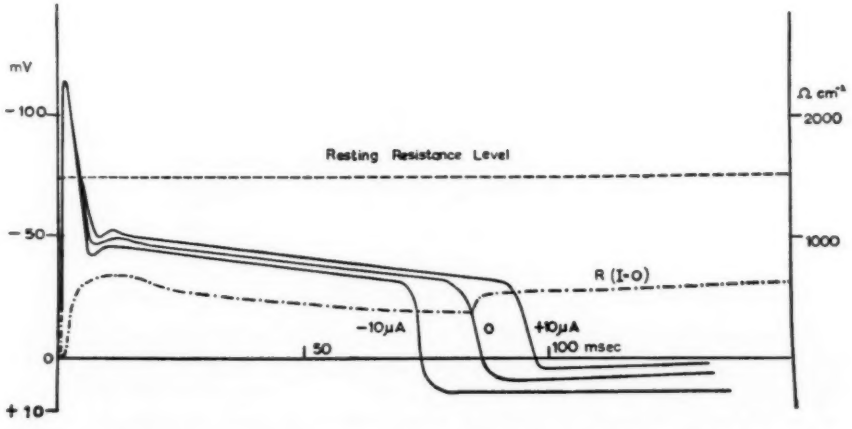

Fig. 3. Effect of constant hyperpolarising (-10 μA. cm.$^{-2}$) and depolarising ($+10$ μA. cm.$^{-2}$) current (I) on the prolonged action potential. The current was switched on at the same time as the stimulus was applied and continued throughout the following action potential. The time course and magnitude of the membrane resistivity (—·—·—·—·—) when $I = 0$ is also shown. TEA axon ($k = 100$) normal sea water.

Abolition.

It will be noted from Fig. 3 that the effect of hyperpolarising currents is to shorten the plateau. This abolition was investigated systematically by inserting short current pulses into equation (1) from $t = 20$ to $t = 20{\cdot}5$ msec.

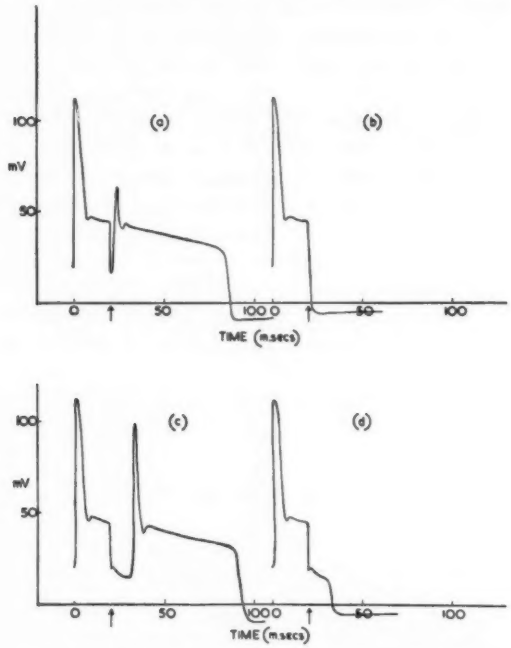

Fig. 4. Effect of short hyperpolarising current pulses (0·5 msec.) of various strengths applied during the plateau of the prolonged action potential (indicated by ↑ in the figure).

a = 80μA. cm.$^{-2}$
b = 90 μA. cm.$^{-2}$
c = 84·452806 μA. cm.$^{-2}$
d = 84·452807 μA. cm.$^{-2}$

The results are shown in Fig. 4. It is seen that for currents less than 80 μA. cm.$^{-2}$, corresponding to a membrane charge (Q) of 0·04 μ coulomb. cm.$^{-2}$ an oscillatory response of short duration ensues, following which the prolonged action potential resumes its original course with minimal disturbance. With $I = 90$ μA. cm.$^{-2}$ or $Q = 0{\cdot}045$ μ coulomb. cm.$^{-2}$, the action potential is abolished. There is a critical value between 84·452806 and 84·452807 μA. cm.$^{-2}$ at which the membrane potential lingers at an unstable level before returning to the original plateau or to the phase of undershoot as the case may be.

Refractory period.

The response to a second stimulus applied at a varying interval after the first stimulus has been investigated and is illustrated in Fig. 5. For intervals greater than 400 msec., the standard stimulus of 20 mV. depolarisation was sufficient to evoke a response. However, for intervals less than 400 msec. the standard stimulus was insufficient. When increased to 40 mV. a response was obtained. Clearly for intervals less than 400 msec., at least, the fibre is in a relatively refractory state. An action potential initiated during this refractory period is shorter than the preceding one. The shortening becomes greater the smaller the stimulus interval. Indeed at the shorter intervals the plateau of the action potential disappears.

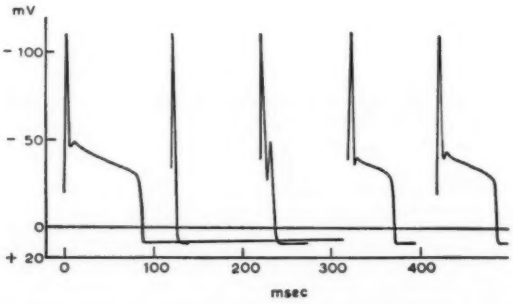

Fig. 5. Demonstration of refractory period following the prolonged action potential. The action potential on the extreme left of the figure is that in response to the first of two stimuli applied at various intervals apart. This first stimulus was a depolarisation of -20 mV. The middle three action potentials are in response to a second stimulus of -40 mV. applied at different intervals after the first. The action potential on the extreme right is in response to a second stimulus of -20 mV. TEA axon ($k = 100$) normal sea water.

The effect of shortening the first action potential by an abolition current pulse is to produce a corresponding shortening of its associated refractory period. This is illustrated in Fig. 6. In calculating this curve a hyperpolarising current pulse of 100 μA. cm.$^{-2}$ was inserted into the computation from $t = 20$ to $t = 20{\cdot}5$ msec. Without abolition, a second 20 mV. stimulus applied 120 msec. after the first is without effect and a 40 mV. stimulus produces an action potential without a plateau. When the first action potential is cut short by the hyperpolarising current pulse, a second 20 mV. stimulus now produces an action potential with a well defined plateau.

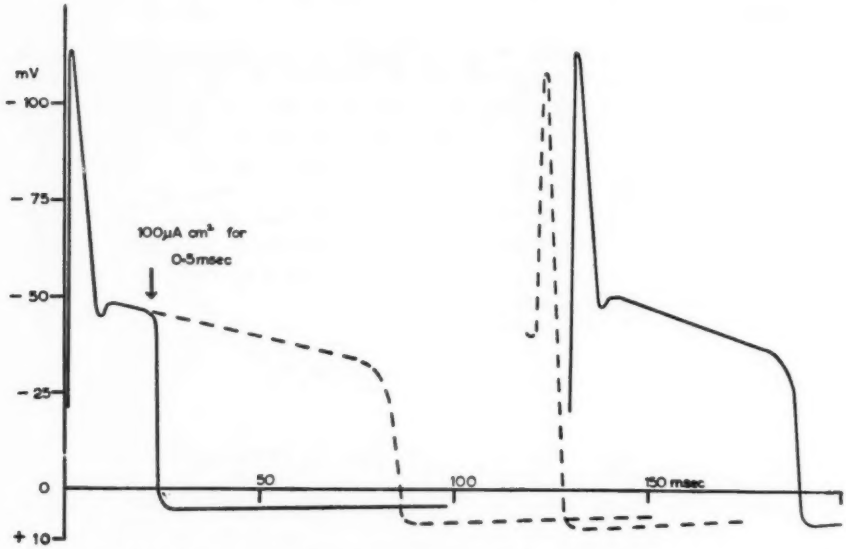

Fig. 6. Effect of abolition on the refractory period. The interrupted line shows the action potential waveforms in response to two stimuli applied 120 msec. apart. The first (− 20 mV.) evokes a prolonged action potential, the second (− 40 mV.) a brief spike. The continuous line shows the waveforms in response to two stimuli when a hyperpolarising current pulse (100 μA. cm.$^{-2}$ for 0·5 msec.) is applied at the time indicated by the ↓ in the figure to abolish the first action potential. The response to the second stimulus of − 20 mV. (drawn displaced in time by + 20 msec. to avoid interference with the other extra response) now evokes a prolonged action potential. TEA axon (k = 100) normal sea water.

Anode break responses.

As an extension of the work on the effect of applied currents, we calculated the time course of the membrane potential for the following current values in equation (1):

$$I = 0,\ t < 0 \text{ and } t > 50 \text{ msec.}$$
$$I = I_1,\ 0 < t < 50 \text{ msec.}$$

For positive values of I_1, this corresponds to the case when a hyperpolarising current pulse of 50 msec. duration is applied to the resting membrane.

The results are shown in Fig. 7 for $I_1 = -10$ μA. cm.$^{-2}$ and $I_1 = 20$ μA. cm^{-2}. During the duration of these hyperpolarising currents, the membrane potential moves to a more positive value in which the sodium carrier mechanism is virtually absent ($m = 10^{-3}$, $h = 1$ and $I_{Na} = 10^{-12}$ amp. cm.$^{-2}$). When the current is switched off at $t = 50$ msec. there is an interval of about 10 msec. during which the potential decays and the sodium carrier mechanism is activated sufficiently to cause a regenerative depolarisation. A typical prolonged action

potential is thus produced similar in every way to those already seen in previous figures. The duration of this action potential increases slightly as the magnitude of the hyperpolarising current used to evoke the anode break response is increased.

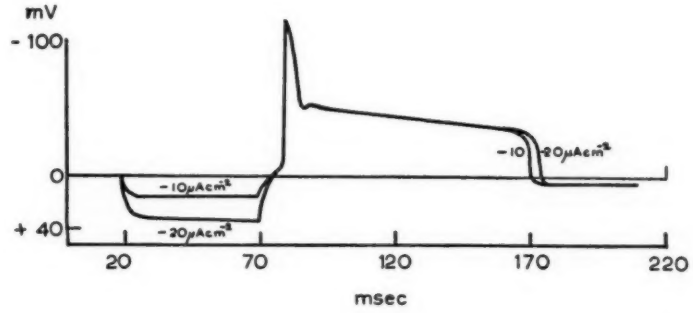

Fig. 7. Anode break response following a hyperpolarising current pulse of -10 μA. and -20 μA. cm.$^{-2}$ and of 50 msec. duration. The current was applied for times between 20 and 70 msecs. on the abscissa scale. TEA axon ($k = 100$) normal sea water.

Potassium-rich Sea Water.

Hyperpolarising responses.

Stämpfli (1958) showed that the frog node of Ranvier displayed two stable states in enriched KCl. A transition between these two states was produced by the application of hyperpolarising current.

Segal (1958) and Tasaki (1959) have shown that when squid axon is placed in potassium enriched media it loses its normal responsiveness to a depolarising current pulse. However, with hyperpolarising currents above a certain threshold intensity the axon develops large "hyperpolarising responses".

Inspection of equation (1) shows that the only modification which is necessary in order for it to hold for a change in the external potassium concentration is for V_K to be altered in accordance with the prescription in (c) on p. 278 above. We solved equation 1 with $k = 1$ (normal axon) and with $V_K = -20$ mV. or $-41{\cdot}6$ mV. corresponding to external potassium concentrations of 46 and 110 mM respectively.

The membrane current was set initially to zero and the mathematical solution allowed to run until a steady state was reached. Then various membrane currents were inserted in the r.h.s. of equation (1). The resulting solutions are shown in Fig. 8a for $[K]_o = 46$ mM, and in Fig. 9 for $[K]_o = 110$ mM.

With $[K]_o = 46$ mM, Fig. 8b shows that small depolarising and hyperpolarising currents result in minor displacements of the membrane potential

which depend in an approximately linear manner on these currents. The membrane resistivity defined as the ratio $(\Delta V/\Delta I)_{\lim_{\Delta V \to 0}}$ in this current range is found to be 100 ohm. cm.^{2} At intermediate hyperpolarising currents small oscillations occur which may be damped (-30 μA. cm.$^{-2}$) or continued (-40 μA. cm.$^{-2}$). At larger hyperpolarising currents shown in Fig. 8a (-45 μA. cm.$^{-2}$) quite large positive displacements occur in the membrane potential, the final steady value again being approximately linearly dependent on the membrane current, but with quite a different value of the ratio $(\Delta V/\Delta I)_{\lim_{\Delta V \to 0}}$ which is now 3300 ohm. cm.^{2}

Clearly between 40 and 45 μA. cm.$^{-2}$ there is a discontinuity in mem-

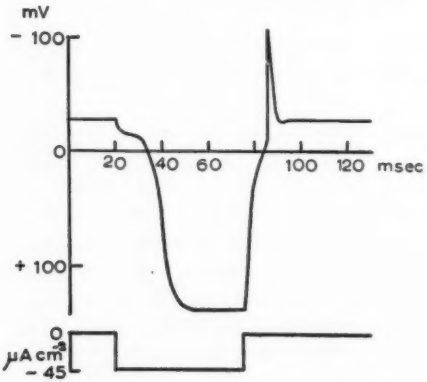

Fig. 8a. Hyperpolarising response in K-rich media (a). Response to a hyperpolarising current pulse of -45 μA. cm.$^{-2}$, and of 50 msec. duration. Note the "step" or "shoulder" in the early part of the response and the anode break response following the termination of the current. Normal axon in K-rich sea water $[K]_o =$ 46 mM.

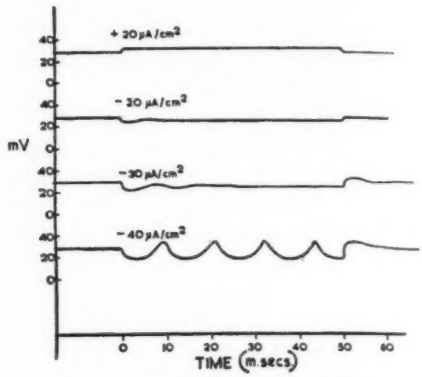

Fig. 8b. Response to small hyperpolarising and depolarising current pulses of 50 msec. duration applied between t = 0 and t = 50 msec.

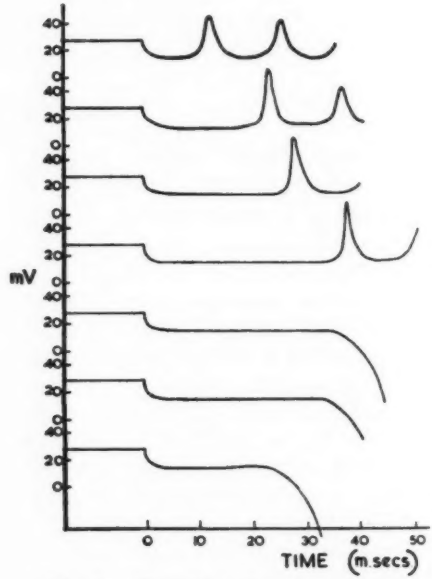

Fig. 8c. The effect of various values of hyperpolarising current of 50 msec. duration and close to the threshold for the hyperpolarising response. From top to bottom currents in μA. cm.$^{-2}$ are 43·0, 43·4, 43·400026, 43·40002618792, 43·40002618794, 43·400026188, 43·400028.

brane resistance which results in a hyperpolarising response. This range of current values was explored in more detail. The results are shown in Fig. 8c. The main effect to be noted is that as the current strength is increased towards the threshold value of 43·40002618794 μA. cm.$^{-2}$ the "step" or "shoulder" on the hyperpolarising response becomes longer before the large positive swing in membrane potential occurs. The solutions have been described in detail for $V_K = 20$ mV., $[K]_o = 46{\cdot}0$ mM. The results in Fig. 9 for $V_K = 41{\cdot}6$ mV., $[K]_o = 110$ mM show a hyperpolarising response similar in form to that for $[K]_o = 46$ mM.

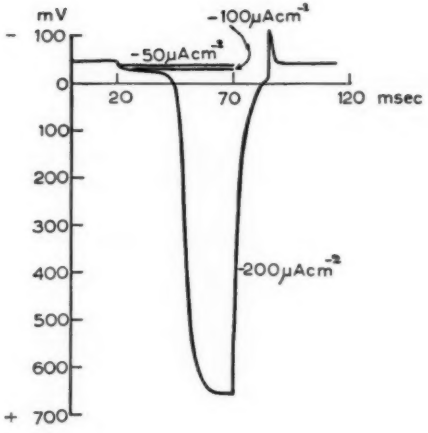

Fig. 9. Hyperpolarising response in K-rich media. Responses to hyperpolarising currents pulses of -50, -100, -200 μA. cm.$^{-2}$ and 50 msec. duration. The current was applied for times between 20 and 70 msec. Normal axon in K-rich sea water, $[K]_o = 110$ mM.

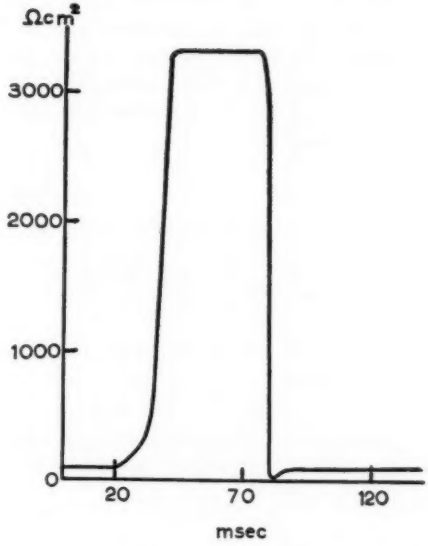

Fig. 10. The time course and magnitude of the membrane resistivity before, during and after a hyperpolarising response. The hyperpolarising current (-45 μA. cm.$^{-2}$) was applied between $t = 20$ and $t = 70$ msec. Ordinate: membrane resistivity in ohm. cm.^{2}, abscissa: time (t) in msec. $[K]_o = 46$ mM.

Anode break responses.

The hyperpolarising currents were switched off at $t = 50$ msec. The anode break responses predicted by the Hodgkin-Huxley equations for these particular conditions are shown in the latter parts of Figs. 8a and 9. The first 10 msec. are occupied with the return of the membrane potential towards its original value and the consequent changes in the activation of the sodium and potassium

carrier mechanisms. The resulting action potential consisted of a brief spike ($k = 1$) of reduced amplitude. The small size of the spike is caused by the low value of the resting potential in the K-rich media. The absolute value of the potential at the crest of the spike is identical to that produced in an axon in normal sea water (-114 mV.).

Resistance values.

The values of membrane resistivity during the hyperpolarising responses for $[K]_o = 46$ mM were computed using equations (11) and (12) and the result for one value of current (45 μA. cm.$^{-2}$) is shown in Fig. 10. Within 20 msec. of the start of the current pulse the membrane resistivity increases from a resting value of 100 ohm. cm.^{2} to 3300 ohm. cm.^{2} At the end of the current pulse, the resistivity falls with a similar delay to a minimum of 25 ohm. cm.^{2} (corresponding to the spike of the anode break response) and thereafter returns with a delay of approximately 10 msec. to the original resting value of 100 ohm. cm.^{2}

DISCUSSION.

Interpretation of bi-stable states of the membrane.

Consider the conditions for equilibrium of a membrane satisfying the Hodgkin-Huxley equations but with the conductivity to potassium ions held constant.

Since, for equilibrium, $dV/dt = 0$, equation (1) becomes, under these conditions:

$$G_K (V - V_K) + \bar{g}_l (V - V_l) + \bar{g}_{Na} m_\infty^3 h_\infty (V - V_{Na}) = 0 \quad \ldots\ldots (13)$$

where G_K is a constant and the other terms have the same meaning as in (1): m_∞ and h_∞ are the asymptotic values of m and h as $t \to \infty$ and are given by

$$m_\infty = \frac{a_m}{a_m + \beta_m} \quad \ldots\ldots \ldots\ldots \ldots\ldots (14)$$

$$h_\infty = \frac{a_h}{a_h + \beta_h} \quad \ldots\ldots \ldots\ldots \ldots\ldots (15)$$

They are determined purely by the magnitude of the membrane potential V (eqns. 7 to 10). The steady values of sodium current given by the last term in equation (13) using (14) and (15) have been evaluated and are drawn in Fig. 11. It is seen that I_{Na} passes through a maximum at $V = -30$ mV. It is zero at $V = V_{Na} = -115$ mV. and is negligible at small positive values of the membrane potential because of the behaviour of $m_\infty^3 h_\infty$.

The first two terms in (13) are linear in V and may therefore be grouped together into one term:

$$G(V - V_o) \quad \ldots\ldots \ldots\ldots \ldots\ldots \ldots\ldots \ldots\ldots \ldots\ldots (16)$$

where $$G = G_K + \bar{g}_l \quad \ldots\ldots \ldots\ldots \ldots\ldots \ldots\ldots \ldots\ldots \ldots\ldots (17)$$

$$V_o = \frac{G_K V_K + \bar{g}_l V_l}{G_K + \bar{g}_l} \quad \ldots\ldots \ldots\ldots \ldots\ldots \ldots\ldots \ldots\ldots \ldots\ldots (18)$$

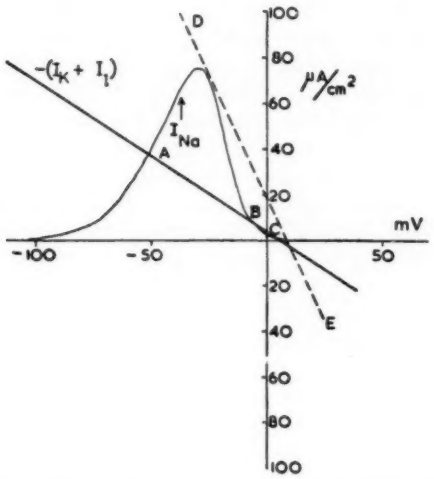

Fig. 11. Graphs of the various current terms in equation (13) for the steady state and where the potassium conductivity is constant at the resting value, $3{\cdot}6\ 10^{-4}$ mhos. cm.$^{-2}$ The solution of equation (13) for this case is given by the intersections of the two full lines. It is seen that two stable states are possible, A and C. For values of G_K greater than the limiting case shown by the interrupted line, only one stable state is possible.

The solution to equation (13) may be obtained by also drawing on Fig. 11 the straight line corresponding to equation (16) but with sign reversed. The intersection of these two lines then gives the solution of equation (13) for V. This has been done with G_K held constant at its resting value of $3{\cdot}6 \times 10$ mhos. cm.$^{-2}$, $g_l = 3 \times 10^{-4}$ mhos. cm^{-2}. It is seen that there are three points of intersection, A, B and C, corresponding to voltages of -52, -6, and $+4$ mV. respectively. Of these, the extreme points A and C are stable solutions. For these points, the membrane behaves as a positive resistance, with an increase in potential bringing about an increase in current. The middle point B is an unstable state since an increase in current causes the point B to move in the negative direction. Thus the point B will not be a stable membrane configuration and we are left with two stable states, A and C.

It is suggested that these two solutions A and C correspond respectively to the "upper" and "lower" of Tasaki's bi-stable states (Tasaki and Hagiwara, 1957; Tasaki, 1959). It can also be readily appreciated from Fig. 11 that with the axon in normal sea water there is a limiting value, G_o, of the potassium conductivity shown as the dotted line DE in Fig. 11. For values of the potas-

sium conductivity in excess of this critical value only one stable configuration of the membrane is possible, namely, the "lower" state C. The critical value of potassium conductivity is found to be $G_0 = 1{\cdot}92 \times 10^{-3}$ mhos. cm^2.

Fig. 11 provides a useful way of looking at many of the results reported in this paper. For example, if G_K increases slowly with time, the straight line (16) rotates slowly in a clockwise direction and at the same time moves slightly to the right as given by equation (18). This means that the "upper" state A moves slowly towards a lower potential, accounting for the slow decrease in potential during the plateau. Finally, the straight line (16) reaches the limiting position, shown interrupted in Fig. 11, and any further increase in G_K means that only one stable state is possible, namely, the "lower" one. Hence the potential must drop quickly to a value close to the point C. This accounts for the recovery and undershoot in the action potential prolonged by TEA.

Applied membrane currents are simply represented by raising the straight line AC (hyperpolarising currents) by an amount corresponding to the applied membrane current. Similarly an applied depolarising current is represented by lowering the line AC by the required amount. In this way it is seen how depolarising currents raise and hyperpolarising currents lower the level of the plateau, and the lengthening and shortening of the action potential by these applied currents is also simply understood in terms of Fig. 11.

Another feature of our results that finds a ready interpretation in terms of Fig. 11 is the sudden appearance of a plateau for values of k in excess of 22·5. In Fig. 12 we have plotted the value of $G_K = \bar{g}_K n^4$ as a function of time for various values of k. As k is increased the curve for G_k becomes flatter but for $k < 22{\cdot}5$, G_K exceeds the critical value G_0 early in the spike phase of the action potential. This returns the membrane to the point C in Fig. 11. For values of k greater than 22·5, the values of G_K is less than G_0 for an appreciable time after the spike giving the membrane potential the opportunity to settle down at the "upper" state A in Fig. 11.

Consider now Fig. 11 redrawn to correspond to conditions of normal axon in potassium enriched sea water. This has been done in Fig. 13. The curve for steady sodium current is the same as in Fig. 11 but the curve of minus one times the potassium current is now quite different. It crosses the horizontal axis more to the left, since V_k (equation (18)) has moved in this direction. Further, for normal axon the relation between V and I_K will be curvilinear as indicated. The effect of depolarisation by increasing the external potassium is seen to result in only one (the "upper") stable state corresponding to A in Fig. 13. Depolarising currents will result in a shift of the point A to the left, but otherwise nothing singular.

With small hyperpolarising currents a similar result follows but when the hyperpolarising current is sufficiently large so that the potassium current curve occupies some such position as that shown interrupted in Fig. 13, it is seen the "upper" stable state is no longer possible. The membrane potential is forced

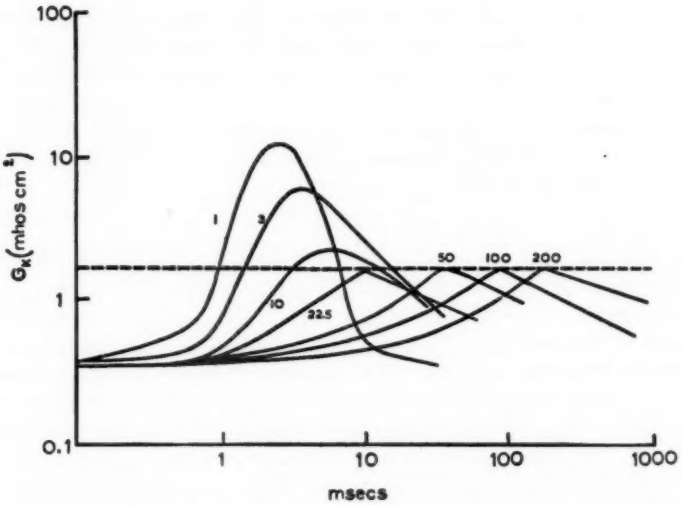

Fig. 12. Time course of G_K for various values of k (the retardation factor) of the potassium-carrying mechanism. The interrupted line shows the critical value of G_K derived from Fig. 11. For values of $k > 22{\cdot}5$, G_K never exceeds this critical value.

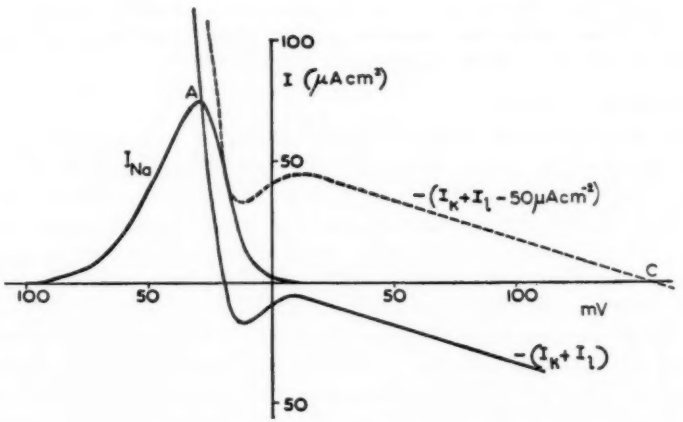

Fig. 13. The separate membrane currents I_{Na} and $-(I_K + I_l)$ in the presence of raised extracellular potassium ($[K]_o = 46$ mM). The solution of equation (13) is given by the intersection of the two solid lines (point A), and this is the only stable state. The situation for added hyperpolarising current of 50 μA. cm.$^{-2}$ is shown by the interrupted line. For I equal to or greater than this value only one stable state is possible corresponding to the hyperpolarised state, point C.

to return to the point C and in this way the hyperpolarising response is accounted for. For intermediate values near the threshold, when the overlap of the two curves is first being abolished, detailed computations show that the two current lines overlap to a small degree and have two points of intersection a few mV. apart. These two points of intersection correspond to the limits of the excursion of oscillatory response noted for hyperpolarising currents close to 40 μA. cm.$^{-2}$ (Fig. 8a, b).

TEA-treated axon.

The authors have been impressed by the ability of the Hodgkin-Huxley equations, modified only to comply with the experimental conditions, to predict the behaviour of squid axon treated with TEA. In all respects, the qualitative nature of the behaviour has been predicted. There are some quantitative discrepancies between predicted and observed behaviour. Inspection of the published records of Tasaki and Hagiwara (1957) shows that the membrane potential at the crest of the spike is somewhat lower than the predicted value of -114 mV. The computed action potentials also show a small notch separating the spike and plateau which is not seen in the experimental records. These discrepancies are not surprising in view of the fact that the numerical data which we used in the computations are those published by Hodgkin and Huxley for their experimental results for a normal axon. It is quite likely that there are differences between these values and those holding for the experiments reported by Tasaki and Hagiwara and that such differences would account for the qualitative discrepancies described above. Further comment would seem unwarranted until the complete voltage clamp data are available for a TEA-treated axon and a full computation carried out.

Hyperpolarising response.

Qualitative agreement at least between computed and experimental results has been obtained for certain features of the hyperpolarising response in K-rich solutions. The equations predict a linear relation between membrane potential and applied current for depolarising and for small hyperpolarising currents. They predict a sudden discontinuity in membrane resistance at a certain threshold value of current, beyond which the membrane resistance assumes a greater value. At threshold and just super-threshold values of hyperpolarising current, the membrane lingers in an unstable region between the two resistance states and the response exhibits a "shoulder" or "step" before the upstroke of the response. At the termination of the current pulse, the potential falls towards the resting potential and an inflexion in the potential waveform occurs before the upstroke of the following anode break response. These are characteristic features of the observations reported by Segal (1958) and Tasaki (1959).

However, the equations do not reproduce the experimentally observed spike in the hyperpolarising response with its associated transient drop in resistance. Indeed, the resistance time course predicted does not show a maintained drop

in membrane resistivity *below* the resting value following the short anode break response. Segal (1958) found that the fibre was relatively refractory to hyperpolarising pulses for 1½ mins. after a response. This feature the equations also do not predict.

We feel, however, that it is perhaps not surprising that the equations fail to predict this behaviour for they were not developed to account for long-term changes in membrane properties. The voltage clamp data for which these equations were formulated did not include observations of membrane current for long durations of voltage clamp. Until such data are available it is not possible to determine whether the equations are incapable of predicting all the features of the hyperpolarising response.

Acknowledgments. We should like to thank Professor H. Messel for providing the excellent facilities of the Adolph C. Basser Computer Laboratory in the School of Physics. We are very grateful to Dr. John Bennett for showing us how to use the fast computer SILLIAC and to the Laboratory Staff for carrying out the many computations required for this paper.

After this investigation was completed our attention was drawn to the paper of Fitzhugh (J. gen. Physiol., June, 1960) in which are presented results, obtained with an analogue computer, covering some of the features of our paper. According to Fitzhugh, an analogue computer had to be used because of the delays experienced in the processing of data with a fast digital computer. As we were invariably able to get our data processed within 24 hours at the most we did not have the same necessity to abandon the more accurate digital computation.

Fitzhugh has introduced a slightly different modification into the Hodgkin-Huxley equations in that one of the sodium mechanisms was accelerated and the potassium carrier mechanism delayed. With a slowing down by a factor of 100 times our results are essentially similar to and confirm those of Fitzhugh. The effects of raising the potassium concentration and hyperpolarising response were not explored by Fitzhugh.

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RECOVERY OF *STRIGOMONAS ONCOPELTI* AFTER DRYING FROM THE LIQUID STATE

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SUMMARY.

The flagellate *Strigomonas oncopelti* has been recovered after drying from the liquid state in a high concentration (50 p.c.) of glucose. Lower concentrations of the sugar are less effective.

Deterioration of the desiccates is influenced strongly by storage temperature, being relatively rapid at room temperature and less so at 4° C. At − 20° C. the desiccates show a high stability and approximately 5 p.c. of the cells exhibit active motility when rehydrated after storage for six months.

INTRODUCTION.

The recovery of the flagellate S. *oncopelti* from dried peptone plugs after storage for twelve months has been described (Annear, 1956a). Subsequently, experiments have shown that this protozoan could be recovered after drying by a simpler method, i.e. from the liquid state as studied with bacteria (Annear, 1958a). Small volumes of heavy suspensions of the flagellate in glucose have yielded, after drying from the liquid state, appreciable numbers of actively motile organisms. In this study measurements have been made of the fall in temperature which occurs during the evaporation of the drops of microbial suspension.

In the earlier work (Annear, 1956a) the organisms were dried in a mixture of peptone and glucose. Some preliminary experiments with the present drying method indicated that there was little, if any, difference whether or not peptone was included in the suspensions. The present studies have therefore been restricted to glucose alone as the preserving agent.

The factors so far which have been studied in relation to the drying and recovery of the organism are glucose concentration, period of dehydration and storage temperature.

METHODS.

Preparation of suspensions for drying.

The organism was cultured in a peptone-glucose medium (Newton and Horne, 1957), usually in 400 ml. volumes in 1 litre Erlenmeyer flasks gently shaken at 28° C. The

cultures when used were young and heavily turbid, but not critically standardized. The cells were centrifuged and washed once in 5 p.c. glucose. The cell deposits were then mixed with approximately twice their volume of more concentrated glucose solutions; the final concentrations of the three glucose levels investigated were approximately 15, 30 and 50 p.c. (w/v). Thick homogeneous suspensions were prepared by stirring with a fine glass rod.

Using a syringe pipette (Annear, 1956b) single drops (0·03 ml.) of the suspension were placed in ampoules (Edwards, E30/C17; Fig. 1) which were plugged with cotton wool. The number of flagellates/drop was approximately $2{\cdot}0 \times 10^8$.

Drying.

The manifolds used were similar in design to that described previously (Annear, 1958a) but were made from copper and brassplated with cadmium. After being charged, the ampoules were connected to the manifold and lowered into a water bath at ambient temperature (ca. 20° C.). They were then exposed to P_2O_5 at high vacuum produced by a Speedivac 2SC50 pump. After 15-30 minutes the ampoules were taken off one by one, constricted and placed on a vertical manifold, again over P_2O_5, where they were sealed after periods ranging from 30 minutes to several days.

Storage.

The ampoules were stored in darkness at either 4° C., − 20° C., or room temperature, which was usually about 20° C., but ranged between 17° C. and 30° C.

Assessment of viability.

In assessing viability for the comparison of treatments, the desiccates were rehydrated and examined by phase microscopy and/or cultured for growth.

Immediately after rehydration the flagellates appear either dead or exhibit varying degrees of activity from sluggish wriggling to vigorous motility. Within about 15 minutes the wrigglers are no longer seen and the cells appear either actively motile or inert. In some experiments attempts have been made to estimate the approximate fraction of cells showing active motility in rehydrated preparations. A total count was made of the inoculum before drying and the motile cells found in the rehydrated preparations were expressed as a percentage of this figure. No attempt has been made to estimate the value below 0·1 p.c. The counts were made in a Neubauer chamber.

In culture, the treatments were compared on the basis of the time taken for rehydrated desiccates to reach a macroscopically recognizable degree of growth, this being a fine granular surface film which shakes down the column of fluid when gently disturbed. The medium used for rehydrating the organisms had the following composition: Lab lemco, 1 p.c.; Evans' peptone, 1 p.c.; glucose, 10 p.c.; Yeastolate (Difco), 0·2 p.c. (w/v). The formula is an empirical one and appeared to give more rapid and consistent recoveries than the medium used to grow the cells for drying but critical comparisons have not been made. The desiccates were usually rehydrated with 5 ml. of medium which was then divided into ten 0·5 ml. aliquots held in small tubes (4 in. × 5/16 in.). This procedure reduced the loss of information which occurs because of contamination in occasional tubes. In some experiments ampoules were rehydrated after removal from the manifold while in others the rehydration was carried out after sealing and storage. The tubes were incubated at 28° C., examined daily and discarded as negative if no growth appeared in 30 days.

In assessing treatment differences by the periods taken for rehydrated organisms to show visible growth precise measurement is made difficult by the occurrence of contaminated tubes and the wide differences which occurred within some treatments. Nevertheless some conclusions are possible from the information available.

Temperature measurement on evaporating suspensions.

Fig. 1 is a diagram of the copper-constantan thermocouple used to measure the temperature of the evaporating drop of suspension. The wires were sealed through the wall of the ampoule with "Araldite" and the current was measured on a Cambridge galvanometer.

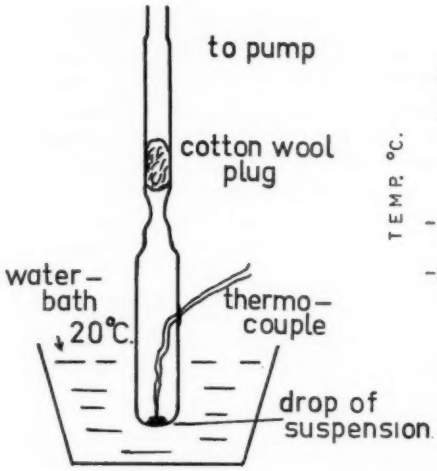

Fig. 1. Assembly for measuring temperature fall during evaporation *in vacuo* of microbial suspension.

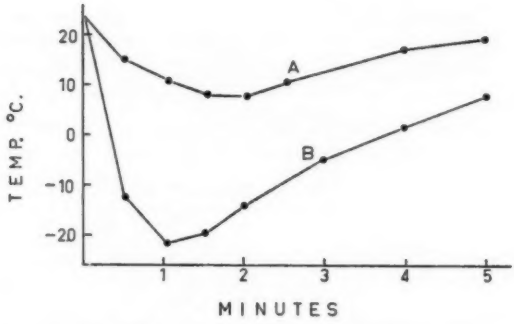

Fig. 2. Temperature readings on drops of fluids evaporating *in vacuo*.

A. Drop (0·03 ml.) of suspension of *Strigomonas oncopelti* in 50 p.c. glucose. For diagram of assembly, see Fig. 1.

B. Drop of distilled water in unplugged ampoule, exposed externally to room atmosphere.

Curve A in Fig. 2 shows the temperature changes which occur under the conditions which gave the highest recoveries in this study. It is apparent that the temperature fall was only a few degrees and the minimum temperature was well above the freezing level. For comparison Curve B shows the temperature changes of a simple system in which freezing does occur during evaporation.

RESULTS.

Effect of glucose on the organism in wet suspensions.

Cells suspended in 50 p.c. glucose solutions appear osmosed and quite lifeless when examined microscopically. Upon dilution of the suspension to a sugar concentration of about 20 p.c. or less the cells regain a normal appearance and are actively motile. This recovery can be observed after storage of the suspensions for several weeks at 4° C. but there is a gradual deterioration.

Effect of glucose on drying.

The highest concentrations of glucose investigated for protection during drying resulted in the highest recoveries (Table 1). There were, as in all ex-

TABLE 1.

Recoveries of Strigomonas oncopelti *after drying in three concentrations of glucose.*

Single drops of suspension in each concentration of glucose were dried in ampoules for 2 hours; the ampoules were then constricted and dried for a further 2 days. They were then sealed and stored at room temperature or 4° C. To determine the recovery ampoules were examined in replicates of three. Each was rehydrated to 5 ml., then divided into $10 \times 0{\cdot}5$ ml. aliquots and incubated for growth tests.

Concentration of glucose p.c. (w/v)	Recoveries after drying 2 days	Recoveries after storage		
		3 months at room temp.	12 months	
			Room temp.	4° C.
15	Approx. half the tubes gave positive cultures in 14-16 days. Remainder no growth	No recoveries		
30	All tubes positive in 4-6 days	All tubes positive in 12-18 days	No recoveries	All tubes positive in 9-11 days
50		All tubes positive 8-10 days	3/30 tubes positive in 20 days. Remainder negative.	

TABLE 2.

Recoveries of Strigomonas oncopelti *after drying for different periods, then storage at room temperature and 4° C.*

Single drops of suspension were dried in 50 p.c. glucose. The ampoules were dried for 30 minutes, constricted, and sealed after the total period of drying indicated in the Table. The recovery was tested as in Table 1.

Experiment	Period of drying (hours)	Period of storage (months)	Temperature of storage	Recovery periods for growth (days)
A	2	4	Room	11-20
			4° C.	7-8
		12	Room	No recoveries
			4° C.	8-10
	24	4	Room	11-20
			4° C.	7-8
		12	Room	No recoveries
			4° C.	9-11
B	1	6	Room	11-15
		12	Room	No recoveries
			4° C.	6-7
	4	12	Room	2/30 tubes positive
			4° C.	6-7
	72	6	Room	11-13
		12	Room	No recoveries
			4° C.	6-7

periments, much higher recoveries from ampoules stored at 4° C. than from those stored at room temperature, and differences have not yet been seen between the two higher concentrations of glucose with these desiccates. The effect, however, is quite marked with the material stored at room temperature.

Period of drying.

Table 2 shows some results from two experiments in which the suspensions were dried for various periods before sealing and storage. With material stored at 4° C. there were no differences between ampoules dried for periods ranging from 1 to 72 hours and no clear differences for material stored at room temperature.

Table 6 shows no differences between recoveries from ampoules which were continuously pumped for 90 days and those sealed after 24 hours pumping and stored for 90 days at room temperature.

TABLE 3.

Relationships between recoveries of Strigomonas oncopelti *and periods of drying, periods of storage and storage temperature.*

Single drops of suspension of flagellates in 50 p.c. glucose were dried as follows:—

Treatment A. Tubes constricted after 30 minutes, then sealed after a further 2 hours on manifold. Stored at −20° C.

Treatments B, C, D. Tubes constricted after 20 hours and sealed after a further 4 hours. B, stored at room temperature; C, stored at 4° C.; and D, stored at −20° C.

Treatment E. Tubes left pumping on manifold at room temperature.

For each treatment at the various periods, 3 tubes were examined to determine the recovery. Each tube was rehydrated to 5 ml. and examined microscopically for motile flagellates. Each tube was then divided into 10 × 0·5 ml. aliquots which were incubated for cultural recoveries.

Treatment	Period on manifold (hours)	Period of storage (days)	Recovery	
			p.c. motile flagellates	Period for visible growth (days)
E	2	—	10	1
	4	—	7	1
	30	—	5	2
	48	—	2	3
	120	—	1	3-4
B	—	14	0·1	4
	—	28	0·1	4-5
A	—	90	5	1-2
B	—	90	S*	4-6
C	—	90	1	2-3
D	—	90	3	1-2
A	—	180	5	1-2
B	—	180	S*	5-7
C	—	180	0·5	2-3
D	—	180	3	1-2

* S=Some actively motile organisms present (below 0·1 p.c.).

Storage temperature.

Tables 1, 2, 3 and 5 all show the marked effects of storage temperature on the desiccates. Material stored at $-20°$ C. (Table 3) has shown no deterioration after six months storage, whereas that stored at 4° C. or at room temperature has shown gradual loss of viability. In many experiments, after prolonged storage marked differences have occurred between desiccates kept at the two higher temperatures (Tables 1, 2 and 5) and in numerous instances, no recoveries have been obtained from material stored at room temperature.

TABLE 4.

Recoveries of Strigomonas oncopelti *after drying and storage at room temperature for* 6 *months.* Single drops of flagellates were dried in 50 p.c. glucose for 24 hours. Recovery tested as in Table 1.

Experiment	Desiccate	Days of incubation for visible growth — Tube									
		1	2	3	4	5	6	7	8	9	10
1	A	7	7	7	7	7	7	8	8	8	11
	B	6	7	7	7	7	7	7	9	10	11
	C	6	7	7	7	7	8	9	9	9	10
2	A	7	10	10	10	10	11	11	11	C	N
	B	10	10	10	10	10	11	11	11	14	17
	C	9	10	10	10	10	10	10	10	11	12
3	A	All tubes positive 5-7 days									
	B										
	C										

C=contaminated.
N=no recovery.

TABLE 5.

Recoveries of Strigomonas oncopelti *after drying and storage at room temperature and* 4° *C. for* 17 *months.*

Single drops of flagellates in 50 p.c. glucose were dried for 24 hours and recovery tested as in Table 1.

Experiment	Temperature of storage	Desiccate	Days of incubation for visible growth — Tube									
			1	2	3	4	5	6	7	8	9	10
1	Room	A	N	N	N	N	N	N	N	N	N	N
	4° C.	A	7	7	9	9	9	9	10	10	11	11
		B	7	7	7	7	8	9	9	10	10	10
2	Room	A	13	N	N	N	N	N	N	N	N	N
	4° C.	A	7	7	7	7	7	7	7	9	9	10
		B	7	7	7	7	7	9	9	10	10	10

N=no recovery.

Variation among results.

Table 4 illustrates the degree of variation that occurs between replicate tubes from the one desiccate, between replicate desiccates from the one experiment and between similar experiments.

TABLE 6.

Relationships between recoveries of Strigomonas oncopelti, *periods of drying and periods of storage.*
Single drops of flagellates in 50 p.c. glucose were dried as follows:
Treatment A. Tubes left pumping on manifold at room temperature.
Treatment B. Tubes constricted after 20 hours, then sealed after a further 4 hours' pumping and stored at room temperature. Recovery tested as in Table 3.

Treatment	Period on manifold (days)	Period of storage* (days)	Recovery	
			p.c. motile flagellates	Period for visible growth (days)
A	1/6	—	10	1
A	1	—	5	1-2
A	2	—	3	2
A	4	—	2	2-3
A	9	—	0·5	3-4
A	90		S	6-7
B		90	S	6-7

* includes initial period on manifold.
S=some motile organisms present.

DISCUSSION.

This work has shown that *Strigomonas oncopelti* may be dried with some success from suspensions in concentrated glucose solutions.

Tables 3 and 6 show that with continuous pumping at room temperature there is a steady fall of motile cells. The results of Tables 2 and 6 also indicate that after about one hour the fall occurs whether the desiccates are on the pump or are sealed and stored at the same temperature, suggesting that a stable moisture level is reached after, at the most, this period of one hour. Approximately 10 p.c. of the cells are motile at this stage and values approaching this level have been maintained only by transferring the desiccates to storage at $-20°$ C. At the two higher temperatures tested deterioration continues much more rapidly at room temperature than at 4° C.

From the results so far obtained, motile cells and positive cultures may be expected from material stored at 4° C. or $-20°$ C. for a number of years.

At room temperature many desiccates have failed to yield living cells after twelve months' storage. However, motile cells have been seen in *some* desiccates after as long as seventeen months. In many cases, positive cultures have been obtained from desiccates in which no motile cells were seen; the age of the

oldest such desiccate is twenty months. Either actively motile cells have been so few as to escape detection or some of the inert or feebly motile cells recover their activity on incubation.

The findings of Leach and Scott (1959) and of Meryman (1960) suggest that the conditions of rehydration may be critical for this organism and increasingly so as the desiccates age. The cells which, when first rehydrated, show some sluggish motility before becoming inert, may respond more favourably to modified conditions of rehydration.

There have been considerable differences in results between experiments done under similar conditions (Table 4) and it is obvious that some factors have not been standardized sufficiently (e.g. the condition of the culture to be dried, numbers of organisms in the inocula and room storage temperature). It is doubtful, however, whether these aspects are worth pursuing until more is known about death on storage and this can be studied by varying the factors within single experiments. The painstaking work of Scott (1959) and his colleagues, and of Greaves (1960) has provided an abundance of information which merits investigation with regard to this aspect of the problem.

In general, and as may be expected, variation is more marked among desiccates on experiments where recoveries are low (e.g. Table 4). Here, where single desiccates have been rehydrated and the apparently homogeneous suspension has been divided into a number of aliquots, some tubes have taken a week or so longer than others to show visible growth and among such tubes occasional ones show no growth at all. Eventually, none or only an occasional tube in a series yields growth (Table 5). Such data are difficult to assess quantitatively and demonstrate the need for considerable replication. There is more homogeneity among tubes from desiccates stored at the lower temperatures, even after prolonged storage (Table 5).

No attempt has yet been made to compare directly the present method of drying *S. oncopelti* with the peptone plug method (Annear, 1956a). The methods are similar in that dehydration is carried out on a highly viscous suspension. Besides differing in the chemical environment, there is also a difference between the physical structures of the two desiccates. With the present method, the suspension dries as a grey bubbly scale on the wall of the ampoule. It is possible that higher and more uniform survival may result by drying the suspension on cellulose fibres as was shown with bacteria (Annear, 1958b).

The concentration of cells in the drops of suspensions dried has been high and would almost certainly contribute markedly to the chemical environment of the desiccates.

Although the present method of drying is reasonably effective for the preservation of *S. oncopelti*, it is unlikely that it will prove, as applied here, of any general use for protozoa. Similar experiments with *Tetrahymena pyriformis* and *Chilomonas paramecium* have yielded completely negative results. On the

other hand, there may be some other protozoa (e.g. Trypanosomes) with which it may be of use.

The initial osmotic dehydration which occurs in the strong sugar solutions does not appear to harm the organisms and it may be that it is a beneficial preliminary step in the drying process and could be borne in mind when investigating other systems.

The results of Meryman (1960) on the drying of the mammalian sperm show how critical the conditions of water removal may be in these processes. Meryman's work and that of Greaves (1960) provide hope of rapid progress in the preservation of the larger and more complex cells by drying.

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